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13. ABSTRACT *(Maximum 200 words)*

In neural crest cells, *bcl-2* overexpression results in altered handling of glutathione, and consequent increased cellular reducing potential. This change potentiates the induction of apoptosis by enediyne prodrugs in neural crest tumor cells. Our studies examined the applicability of these findings to MCF-7 breast cancer cells, known to overexpress bcl-2 in response to estradiol treatment. We have found that estradiol treatment of MCF-7 cells does indeed result in an increase in the expression of bcl-2. However, transfection of MCF-7 cells with an expression construct for bcl-2 does not result in potentiation of enediyne-induced apoptosis. Unlike the case for neural crest tumor cells, glutathione handling is not altered in MCF-7 cells induced by transfection to overexpress bcl-2. Our results to date suggest that determination of the effect of bcl-2 expression on glutathione handling may allow prediction of the response of a particular tumor cell to treatment with enediynes. In the one-year extension period of this grant, we will merge these two lines of investigation and determine the effects of estradiol treatment (rather than direct bcl-2 transfection) on glutathione handling in MCF-7 cells. Initial studies indicate that estradiol treatment does alter the native glutathione content of MCF-7 cells.

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Nina F. Schor 6/29/99

PI - Signature Date

(4) TABLE OF CONTENTS

(5) INTRODUCTION

The proto-oncogene *bcl-2* is expressed in 65-80% of human breast cancers (1,2). Approximately 50% of breast cancers are found to overexpress this gene (1,3). Overexpression of *bcl-2*, which results in overproduction of the 26 kD protein Bcl-2, has been shown to confer resistance to apoptotic cell death induced by a variety of stimuli, including chemotherapeutic-induced apoptosis in breast cancer (4,5). This is particularly important in the case of advanced, estrogen receptor-positive tumors, a circumstance in which Bcl-2 content correlates with clinical resistance to chemotherapy (5).

Recent studies in other cell lines have suggested that Bcl-2 exerts its protective effects by a mechanism that includes a shift in the redox potential of the cell to a more reduced state (6,7). Among other biochemical effects, these studies specifically implicate an increase in cellular reduced glutathione (GSH) content in this alteration in redox potential. This makes particular sense because other studies link the apoptotic process to exposure to reactive oxygen species (8-10). One potential strategy for overcoming Bcl-2-mediated chemotherapeutic resistance is to take advantage of the increase in cellular GSH and, therefore, free sulfhydryl content by using chemotherapeutic agents that require reduction by sulfhydryl compounds for their activity. We have recently explored *in vitro* the efficacy of one such group of agents, the enediynes, in pheochromocytoma cells that were genetically engineered to overexpress *bcl-2* (11).

Neocarzinostatin (NCS) is an enediyne DNA-cleaving natural product that induces apoptosis in some tumor cell lines in culture (12). Like other naturally-occurring enediynes, NCS is actually a prodrug that requires sulfhydryl activation for efficacy. As such, the cytotoxicity of NCS has been demonstrated to vary directly with the sulfhydryl content of the cell (13-15). This information led to our prediction that, contrary to the case for all other chemotherapeutic agents studied, overexpression of *bcl-2* and the resulting shift in redox potential of the cell would potentiate the induction of apoptosis by NCS.

We have shown that, in PC12 rat pheochromocytoma cells that have been *bcl-2*- or control-transfected, *bcl-2* overexpression does indeed potentiate the apoptosis-inducing activity of NCS by increasing cellular levels of GSH (11). The reduction-dependent enediyne prodrugs are therefore the only class of drug that has been demonstrated to work best in those tumor cells that have become resistant to other known chemotherapeutic agents.

Although the naturally-occurring enediynes have been difficult to implement clinically because of toxicity issues (16), recent advances in pharmacology and pharmaceutics have begun to get around these problems. Modifying the NCS chromophore structure to produce other more efficacious and/or less toxic enediynes (17), masking the protein component with inert polymers (18), using enediynes adjunctively with cell-selective activating agents (13), and coupling the newer enediynes to monoclonal antibodies directed at tumor-specific antigens (19) have all proved useful in producing enediyne candidates for human therapeutics. In fact, the last of these

approaches is now in Phase I/II clinical trials for ovarian carcinoma (L. Hinman, personal communication), and has shown promise in a breast cancer cell line in culture (19). The 4-fold gap in sensitivity to NCS that we observed between *bcl-2*- and control-transfected PC12 cells (11) has led us to speculate that the enediynes would be both more efficacious and safer in these patients than in those whose tumors do not overexpress *bcl-2*.

These interesting and promising preliminary results led us to predict that breast cancer cells that overexpress *bcl-2* would have a higher GSH content and therefore be more susceptible to the effects of the enediynes than are breast cancer cells that do not overexpress this gene or normal cells. We further predicted that we could augment the difference in enediyne sensitivity between *bcl-2*-native normal and -overexpressing tumor cells by increasing the availability of cysteine, the rate-limiting substrate for GSH synthesis. Furthermore, we predicted that by increasing the Bcl-2 content of estrogen receptor-positive breast cancer cells with tamoxifen (20) or estrogen (4), we could increase the effectiveness of enediynes against these cells, raising the possibility of adjunctive treatment with enediynes and tamoxifen. This would translate clinically into an improved therapeutic index for treatment of breast cancer with the enediynes.

(6) BODY

(6)-1. Experimental Methods, Assumptions, and Procedures

All of the methods we proposed for use in these studies are currently established in our laboratory using cultured cell systems (11,12,21-29). We describe below the specific hypotheses that were tested and the methods that were used over the two years of Department of Army funding.

(6)-1.1: General Methods Applicable to All Tasks and Technical Objectives

MCF-7 (estrogen receptor-positive) cells were obtained from the American Type Culture Collection (Rockville, MD), and were maintained as stock cultures in α-MEM (GIBCO-BRL) containing phenol red and supplemented with nonessential amino acids, 0.3% glucose, 5% fetal bovine serum, and 2 μg/ml gentamicin sulfate (37°C, 5% CO_2). Where indicated, studies were conducted under four sets of conditions known to alter *bcl-2* expression in this cell line in predictable ways: maintenance of the conditions of the stock cultures (E+; *bcl-2*-positive at "resting" levels; 4,5); addition to the stock culture conditions of 1 nM 17ß-estradiol and maintenance in the estradiol-enriched medium for 48 hr prior to study (E++; 3-fold enhancement of *bcl-2* content over resting levels; 5); maintenance for 7 days prior to study in phenol red-free DMEM (GIBCO-BRL) containing 5% fetal bovine serum stripped of steroids by absorption to dextran-coated charcoal (Sigma Chemical Corp., St. Louis, MO) for 45 min at 45°C (E-; 6-fold reduction of *bcl-2* levels relative to resting state; 4). In addition, E+ cells were studied after a four-day exposure to tamoxifen (10^{-6}M; condition T+), a condition the *in vivo* analogue of which is associated with induction of *bcl-2* (20). In all cases, the same lot of fetal bovine serum (GIBCO-BRL) was used for all conditions in each experiment, and to the extent possible, for all experiments.

In addition, clonal transfectants of MCF-7 human breast cancer cells were obtained from Drs. Charles Rudin and Craig Thompson (University of Chicago, Chicago, IL). These transfectants were produced by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in ref. 30), respectively.

(6)-1.2: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on *bcl-2* content of MCF-7 cells]

For all studies, relative *bcl-2* content of native MCF-7 cells maintained under each condition was assayed by Western blotting as we have previously described (11). In the case of MCF-7 transfectants, stably transfected clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology).

(6)-1.3: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

To determine the GSH and total glutathione (GSH+GSSG) contents of MCF-7 cells containing different amounts of *bcl-2*, E+, E++, E-, and T+ cells (10^7 of each) were washed free of medium, and suspended in 1 ml of phosphate-buffered saline (PBS). The suspension was homogenized and assayed for GSSG and GSH+GSSG by the method of Tietze (31) or for GSH by the Thio-Glo method (32). For the Tietze method, the rate of change in the OD_{412} was measured spectrophotometrically over a period of 3 min. This rate was converted to the total glutathione concentration by plotting on a simultaneously run standard curve for ΔOD_{412} versus total glutathione concentration (constructed using GSH standard solutions). The concentration of GSSG alone was determined by an identical procedure performed on samples that were treated with N-ethylmaleimide prior to assay, to eliminate the reaction of GSH with DTNB (31). The cellular contents of GSH and GSH+GSSG were then calculated for each condition. Each determination was performed in triplicate. Values so obtained for each experiment were compared between E+ and E++, E-, or T+ cells using Student's t test, as we have done in our published work (24,25).

(6)-1.4: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

An NCS concentration-response study was conducted for E+, E++, E-, and T+ MCF-7 cells and MCF-7 transfectants, as we have previously described for pheochromocytoma cells (11). Cells plated in 6-well tissue culture plates were treated with a range of concentrations of NCS (0-0.5 μg/ml) for 1 hr at 37°C. Cultures were then washed free of NCS, and adherent cell number was determined daily in control- and NCS-treated E+, E++, E-, and T+, and MCF-7 transfectant cultures as we have previously described for neuroblastoma and pheochromocytoma cells (11,21,33). The statistical significance of differences between E+ and E++, E-, and T+ cells, in turn, was assessed for each concentration of NCS using Student's t test, with p maximally 0.05 being considered significant.

(6)-1.5: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript co-authored by Drs. Schor, Kagan and his laboratory colleagues, and Thompson and his laboratory colleagues has been submitted to *Molecular Pharmacology* (34). The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

(6)-1.6: Tasks 5, 6 (Determination of glutathione metabolic enzyme activities and efflux rate in MCF-7 cells with varying expression of *bcl-2*)

Our finding that the alteration of *bcl-2* expression by transfection of MCF-7 cells did not lead to changes in glutathione content led to our decision that completion of these Tasks was not likely to be fruitful. We have only recently overcome initial technical

difficulties examining the glutathione content of E-, E+, and E++ cells (see 1998 Annual Report), and have determined that E2 exposure, with its concomitant increase in Bcl-2, leads to an increase in cellular glutathione content that does not appear to be concentration-dependent (38). We have therefore applied for and been granted a one-year no-cost extension to this grant, so that we may complete these studies in this originally-planned model system.

(6)-1.7: Task 7 (Accomplishment of the loading of the membranes of E, E+, E++ cells with cis-parinaric acid)

The membranes of E-, E+, and E++ MCF-7 cells were loaded with *cis*-parinaric acid (PnA; Molecular Probes, Eugene, OR) as we have previously described (32), as an *in situ* probe for the peroxidation of membrane phospholipids. Upon peroxidation, PnA loses its fluorescence, making the fluorescence of each of the membrane phospholipids into which it has been incorporated a measure of the peroxidation of those phospholipids (35).

(6)-1.8: Task 8 (Determination of the susceptibility of E-, E+, and E++ MCF-7 cells to GSH oxidation membrane lipid peroxidation)

Our previous studies have demonstrated that the diazo radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN; Polysciences, Inc., Warrington, PA) induces the concentration-dependent incidence of apoptosis in PC12 pheochromocytoma cells, and that Bcl-2 abrogates apoptosis induction and membrane phospholipid oxidation in these cells (32). While such oxidation in general may accompany or be etiologic in the induction of and commitment of a cell to apoptosis, the specific oxidation and translocation of phosphatidylserine appear to accompany the enactment phase of the apoptotic process (36,37). The pattern of oxidation of individual phospholipids can therefore suggest the point at which reactive oxygen species and their modification play a role in the incidence and/or abrogation of apoptosis. In order to test the hypothesis that the mechanistic relationship between the antioxidant and anti-apoptosis effects of estradiol depends on the duration of estradiol exposure, we have examined the pattern of oxidation of phospholipids in the membranes of MCF-7 cells by AMVN after the addition of estradiol to the medium immediately or for 14 days preceding AMVN treatment.

In the case of immediate estradiol treatment, the medium was removed and cells were maintained for 24 days in estrogen-free, phenol-free DMEM (Biofluids) supplemented with 5% charcoal-treated FBS (Cocalico Biological, Inc., Reamstown, PA). Estradiol was then added at the time of AMVN treatment. In the case of long-term estradiol treatment, prior to each experiment, the medium was removed and replaced with estrogen-free, phenol-free DMEM. The cells were maintained in this estrogen-free medium for 10 days. Subsequently, this medium was replaced with estrogen-free medium supplemented with varying concentrations of 17β-estradiol (E2; 0 - $5x10^{-7}$ M; Sigma, St. Louis, MO), and the cells were maintained in the supplemented medium for an additional 14 days.

PnA served as an *in situ* probe for phospholipid oxidation, as we have previously described (32,35). GSH was measured as detailed above [section (6)-1.3].

(6)-1.9: Task 9 (Preparation of a manuscript describing the findings relative to Tasks 5-8)

A manuscript describing these findings has been accepted for publication in *Biochemical Biophysical Research Communications* (38). The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

(6)-1.10: Task 10 (Determination of the effects of *bcl-2*-overexpression in MCF-7 cells on the increase in glutathione afforded by incubation with N-acetylcysteine)

N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 0.5 - 4 hr. Cells were washed free of extracellular N-acetylcysteine and assayed at various timepoints for total glutathione content by the spectrophotometric method of Tietze (31).

(6)-1.11: Task 11 (Determination of the effects of *bcl-2*-overexpression in MCF-7 cells on the change in sensitivity to NCS afforded by incubation with N-acetylcysteine)

N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 4 hr. Cells were washed free of extracellular N-acetylcysteine (to ensure that NCS did not get activated by this compound extracellularly), and an adherent cell number concentration-response curve to NCS was constructed for each transfectant as we have done in our previously published studies (11). The ED_{50} for NCS was determined for each transfectant as we have previously done for apoptosis induction in SH-SY5Y cells (12,22). The ED_{50}s obtained with and without N-acetylcysteine were compared for each transfectant using Student's t test, as we have previously described (22).

(6)-1.12: Task 12 (Preparation of a manuscript describing the findings relative to Tasks 10-11)

The results of these studies form an integral part of the abovementioned manuscript submitted to *Molecular Pharmacology* (34).

(6)-2. Results and Discussion

(6)-2.1: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on *bcl-2* content of MCF-7 cells]

We have measured the effects of addition to and subtraction from the medium of estradiol to the Bcl-2 content of MCF-7 cells in culture. Our first experiments involved using complete medium as the control, and adding various amounts of estradiol to the

medium, as originally described in the proposed Methods. These studies, all using the same batch of serum for constitution of the medium, demonstrated no change in the Bcl-2 content of the cells. Our assumption was that the serum-replete medium contained suffient estradiol that neither our addition of small amounts of this compound nor our partial depletion of estradiol with charcoal treatment made a significant change in the Bcl-2 content. We surmounted this problem in two ways. First, we obtained estrogen-free medium commercially stripped (by column chromatography) of estrogen, and performed our studies by adding back various amounts of estradiol. This study conclusively demonstrated the estrogen concentration-dependent increase in concentration of Bcl-2 in the cells (See Figure 1). In addition, we have obtained from the laboratory of Dr. Craig Thompson (University of Chicago, Chicago, IL) two clones of MCF-7 cells that have been transfected with the *bcl-2* gene, and their mock-transfected counterparts. We have confirmed overproduction of Bcl-2 in the *bcl-2*-transfected cells, and have maintained all of these lines in our laboratory.

(6)-2.2: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

In the first year of funding, we measured the total glutathione content of the transfectants obtained from Dr. Thompson's laboratory. Accompanying the increase in Bcl-2 content afforded by the *bcl-2* transfection, there was a small but significant increase in glutathione content (see Figure 2).

Recently, we have been able to maintain MCF-7 cells in estrogen-depleted (E-) medium, medium with a physiological estrogen concentration (E+), and media containing each of two estrogen-excess concentrations (E++). With our co-investigators in the laboratory of Dr. Valerian Kagan, we have demonstrated that long-term E2 incubation leads to an increase in total glutathione content of the cells (see Figure 3).

(6)-2.3: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

Our previous studies have demonstrated the paradoxical increase in sensitivity of PC12 rat pheochromocytoma cells to NCS afforded by *bcl-2* transfection (11). This potentiation of NCS toxicity was abrogated by prior incubation of PC12 cells with BSO. To determine the generalizability of these findings to the overexpression of *bcl-2* in different biological systems, similarly transfected MCF-7 human breast cancer cells were examined for the effects of *bcl-2* overexpression on sensitivity to NCS. Unlike PC12 cells, *bcl-2* overexpression protected MCF-7 cells from death induced by NCS (Figure 4). At each concentration, the survival of the *bcl-2* transfectants exceeds that of the mock transfectants.

Attempts to perform the same set of studies in cells exposed to E++, E+, E-, and T+ media, or E- medium and subsequent transfer to graded E concentrations were thwarted by the tendency of estrogen-deprived cells to lift off of the culture surface over the final 5-10 days of the experimental exposure. Although biochemical studies (e.g., glutathione

measurements) can be made on these detached cells, the serial assessment of cell culture growth is technically not feasible using our current techniques.

(6)-2.4: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript describing these findings has been submitted to *Molecular Pharmacology* (34). This manuscript is co-authored by NF Schor, C Rudin, A-R Hartmann, CB Thompson, Y Tyurina, and VE Kagan. The support of the Department of the Army Breast Cancer Program has been acknowledged in this manuscript, and it is appended to this report.

(6)-2.5: Tasks 5, 6 (Determination of glutathione metabolic enzyme activities and efflux rate in MCF-7 cells with varying expression of *bcl-2*)

We plan to perform these studies in the originally described model system in the no-cost extension year of grant funding (see above).

(6)-2.6: Task 7 (Accomplishment of the loading of the membranes of E, E+, E++ cells with cis-parinaric acid)

Specific incorporation of PnA into membrane phospholipids of MCF-7 cells is shown in Table 1. PnA served as an *in situ* probe for phospholipid oxidation, as we have previously described (32,35).

(6)-2.7: Task 8 (Determination of the susceptibility of E-, E+, and E++ MCF-7 cells to GSH oxidation membrane lipid peroxidation)

Treatment of control MCF-7 cells with AMVN (500 μM; 2 h at 37°C) resulted in statistically significant peroxidation of all four phospholipid species examined (Table 2). The fraction of membrane phospholipid peroxidized varied slightly from 3.8% [for phosphatidylethanolamine (PE)] to approximately 2% [for phosphatidylinositol (PI)] of the native total phospholipid content. Phosphatidylcholine (PC) and phosphatidylserine (PS) demonstrated intermediate degrees of peroxidation. This oxidation occurred without sufficient change in the overall phospholipid composition of the membranes to induce necrotic death of the cells (Table 3).

Immediate E2 exposure of MCF-7 cells resulted in statistically significant protection from AMVN-induced peroxidation of PI and PS, and a trend towards such protection of PE and PC (i.e., decreased mean peroxidized fraction after AMVN exposure relative to non-E2-exposed cells that did not reach statistical significance; Figure 5A). This wholesale protection of all phospholipids studied from peroxidation suggests a direct antioxidant role for estrogen in this system.

On the other hand, long-term E2 exposure resulted in the statistically significant protection of PS and, at the highest E2 concentration only, PE (Figure 5B). This pattern is suggestive of Bcl-2-mediated protection from enactment of apoptosis (36,37), rather

than direct protection from membrane phospholipid peroxidation *per se*. Protection of PS in this paradigm was accompanied by a 2-fold decrease in the percentage of the cells demonstrating apoptotic morphology after a 24 h exposure to AMVN (500 μM). Our previous studies demonstrated that apoptotic morphology peaks at 24 h in AMVN-treated PC12 cells (32). Apoptosis was seen in 40 ± 4 (SEM) percent of estrogen-deprived cells exposed to AMVN, and 22 ± 2 percent of long-term E2-treated ($5x10^{-7}$ M) cells similarly exposed ($p < 0.01$, Student's t test).

In light of the previous reports of glutathione-dependent (39) and independent (40-42) effects of E2 and Bcl-2 on apoptosis, we examined the effect of immediate and long-term E2 exposure on the glutathione content of MCF-7 cells. We also determined the effect of AMVN on the glutathione content of immediate and long-term E2-exposed cells. As is shown in Figure 3, immediate E2 exposure had no effect on the glutathione content of MCF-7 cells, while long-term exposure resulted in an increase in glutathione levels. At the concentrations of E2 used, this increase was not concentration-dependent. Regardless of the length of E2 exposure, however, AMVN treatment did not result in a decrement in glutathione levels, indicating that despite the increase in glutathione content that accompanies long-term E2 exposure, the protective effects of such exposure are likely glutathione-independent. This is in concert with previous reports of increased glutathione content related to *bcl-2* overexpression, and the independence from glutathione of the effects of Bcl-2 on apoptosis (6).

These results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (39), but may be enhanced by induction of estrogen metabolism (43). In contrast, *bcl-2* induction associated with long-term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (4). The present studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechnisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.

(6)-2.8: Task 9 (Preparation of a manuscript describing the findings relative to Tasks 5-8)

A manuscript describing these findings has been accepted for publication in *Biochemical Biophysical Research Communications* (38). The co-authors of this manuscript are Drs. Schor, Kagan, Tyurin, and Tyurina. The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

(6)-2.9: Task 10 (Determination of the effects of *bcl-2*-overexpression in MCF-7 cells on the increase in glutathione afforded by incubation with N-acetylcysteine)

Treatment of mock- and *bcl-2*-transfected MCF-7 cells with N-acetylcysteine resulted in GSH content kinetics that resembled those seen with mock-transfected PC12 cells (Figure 2). There was no significant difference between the MCF-7 *bcl-2*- and mock-transfectants in this regard.

(6)-2.10: Task 11 (Determination of the effects of *bcl-2*-overexpression in MCF-7 cells on the change in sensitivity to NCS afforded by incubation with N-acetylcysteine)

In contrast to the case for *bcl-2*-transfected PC12 cells, previously shown by our group to increase in sensitivity to NCS after incubation with N-acetylcysteine, the concentration-response curve of *bcl-2*-transfected MCF-7 cells to NCS is unaffected by N-acetylcysteine treatment (Figure 6). The sensitivity to NCS of mock-transfected cells is similarly unaffected by N-acetylcysteine exposure (data not shown). These results are in accord with our finding that *bcl-2*-transfected MCF-7 cells do not demonstrate altered handling of glutathione.

(6)-2.11: Task 12 (Preparation of a manuscript describing the findings relative to Tasks 10-11)

The results of these studies form an integral part of the abovementioned manuscript submitted to *Molecular Pharmacology* (34).

(7) KEY RESEARCH ACCOMPLISHMENTS

- Determination of the effects of bcl-2 overexpression on the GSH content of MCF-7 breast cancer cells.
- Determination of the effects of bcl-2 overexpression on the susceptibility of MCF-7 breast cancer cells to NCS-induced apoptosis
- Determination of the mechanistic differences between MCF-7 and PC12 cells that determine the differential response to bcl-2 overexpression vis-à-vis GSH handling and susceptibility to NCS-induced apoptosis.

(8) REPORTABLE OUTCOMES

Manuscripts

Schor, N.F., Tyurina, Y.Y., Fabisiak, J.P., Tyurin, V.A., Lazo, L.S., Kagan, V.E. Selective oxidation and externalization of membrane phosphatidylserine: bcl-2-induced potentiation of the final common pathway for apoptosis. Brain Research, in press, 1999.

Schor, NF, Tyurina, YY, Tyurin, VA, Kagan, VE. Differential membrane antioxidant effects fo immediate and long-term estradiol treatment of MCF-7 breast cancer cells. Biochem. Biophys. Research Commun., in press, 1999.

Schor, NF, Rudin, CM, Hartmann, A-R, Thompson, CB, Tyurina, YY, Kagan, VE. Bcl-2-induced alteration of glutathione handling and its exploitation in targeted cancer chemotherapy. Molec. Pharmacol, submitted, 1999.

(9) CONCLUSIONS

Our results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (39), but may be enhanced by induction of estrogen metabolism (43). In contrast, *bcl-2* induction associated with long-term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (4). Our studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechnisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.

Some studies of the effects of *bcl-2* overexpression on ROS handling in neural cells have suggsted a role for Bcl-2 in increasing the tolerance of such cells to oxidant stress (6,7,32). Conversely, some studies have proposed a pro-oxidant role for Bcl-2, and view the enrichment of the intracellular reducing potential as a compensatory, cell-generated phenomenon (44). Still others have pointed out the Bcl-2 overproduction is protective from apoptosis under near-anaerobic conditions (45), and that GSH depletion does not abolish the protective effects of Bcl-2 (43). The present results demonstrate the cell-dependence of the effects of Bcl-2 on GSH metabolism. The impact of Bcl-2 on the reducing potential of the cell is millieu-dependent. This may contribute to the variability of the involvement of ROS and their scavenging in the enactment and prevention of apoptosis.

The determination by the intracellular environment of the redox activity of Bcl-2 and/or the species that are produced or altered as a result of its expression is not unique. The potential for other redox-active agents, including vitamins C and E, to either potentiate or inhibit oxidation is well documented (46).

Our studies of NCS treatment of PC12 pheochromocytoma and MCF-7 breast cancer cells demonstrate the consequences of this biochemical variability and exemplify the potential therapeutic importance of our findings. We have previously reported that, unlike the case for other chemotherapeutic agents from which cells are protected by Bcl-2 (4,5,47-51), Bcl-2 potentiates the induction of apoptosis by the enediyne NCS (11). From the mechanistic standpoint, the present results suggest increased intracellular activation of NCS in *bcl-2*-overexpressing cells, as evidenced by increased production of the glutathionyl radical. The relatively small concentration ratio of added NCS in the bathing medium to GSH intracellularly may be deceptive, since NCS is actively taken up into cells by endocytosis and may therefore be considerably more concentrated intracellularly than extracellularly. Additional increments in NCS concentration may arise from specific compartmentalization of this compound within cellular organelles (52). In addition, our finding that the rate of turnover of GSH is higher in Bcl-2-overproducing PC12 cells than in native producers exposed to NCS implies that not only the endogenous rate of production of ROS, but also the role of GSH in and ability to

compensate for consumption of reducing equivalents is aberrant in some *bcl-2*-overexpressing cells. This increased GSH turnover, as evidenced by increased formation of the glutathionyl radical, is not related to changes in GSH peroxidase activity, as such changes are not associated with glutathionyl radical formation (53). They are rather related to the non-enzymatic generation of glutathionyl radical via interaction with NCS (54,55). This finding, along with our previous observation that the downstream block in the apoptosis final common pathway produced by Bcl-2 is lifted in NCS-treated cells (37), explains the enhanced apoptotic rate in bcl-2-transfected PC12 cells treated with the reduction-dependent prodrug, NCS. The exploitation by NCS of the effects of Bcl-2 on GSH handling in some cells make NCS a potential chemotherapeutic drug for these chemoresistant tumors. However, the cell-dependent effects of Bcl-2 on GSH handling imply that Bcl-2 content alone could not be used as a criterion for predicting the efficacy of NCS against tumor cells.

That incubation with N-acetylcysteine accentuates the difference in GSH handling between *bcl-2*- and mock-transfected PC12 cells, but not MCF-7 cells, suggests that such incubation would augment the potentiation of apoptosis in *bcl-2*-overexpressing PC12 cells, but not in the analogous MCF-7 cells. Indeed, our results bear this out, suggesting that an in vitro assay of GSH accumulation after N-acetylcysteine incubation might be developed to predict the likely responsiveness of a particular tumor to NCS.

(10) REFERENCES

1. Troncone, G, Zeppa, P, Vetrani, A, D'Arcangelo, A, Fulciniti, F, DeDivitiis, B, Palombini, L. bcl-2 protein breast cancer cells obtained by fine needle aspiration (FNA): a preliminary report. Cytopathology 6:219-225, 1995.

2. Doglioni, C, DeiTos, AP, Laurino, L, Chiarelli, C, Barbareschi, M, Viale, G. The prevalence of bcl-2 immunoreactivity in breast carcinomas and its clinicopathological correlates, with particular reference to oestrogen receptor status. Virchows Arch 424:47-51, 1994.

3. Joensuu, H, Pylkkanen, L, Toikkanen, S. Bcl-2 protein expression and long-term survival in breast cancer. Am J Pathol 145:1191-1198, 1994.

4. Teixeira, C, Reed, JC, Pratt, MA. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Research 55: 3902-3907, 1995.

5. Bonetti, A, Zaninelli, M, Pavanel, F, Sperotto, L, Molino, A, Pelosi, G, Cetto, GL, Biolo, S, Piubello, Q. Bcl-2 expression is associated with resistance to chemotherapy in advanced breast cancer. Proc Amer Assoc Cancer Research 37:192, 1996.

6. Kane, DJ, Sarafian, TA, Anton, R, Hahn, H, Gralla, EB, Valentine, JS, Ord, T, Bredesen, DE. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science 262:1274-1277, 1993.

7. Albrecht, H, Tschopp, J, and Jongeneel, CV. Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-kappa B by TNF. FEBS Lett 351:45-48, 1994.

8. Ratan, RR, Murphy, TH, and Baraban, JM. Macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione. J. Neurosci. 14:4385-4392, 1994.

9. Sandstrom, PA, Mannie, MD, and Buttke, TM. Inhibition of activation-induced death in T cell hybridomas by thiol antioxidants: oxidative stress as a mediator of apoptosis. J. Leukoc. Biol. 55:221-226, 1994.

10. Forrest, VJ, Kang, YH, McClain, DE, Robinson, DH, Ramakrishnan, N. Oxidative stress-induced apoptosis prevented by Trolox. Free Radic Biol Med 16: 675-684, 1994.

11. Cortazzo, M and Schor, NF. Potentiation of enediyne-induced apoptosis and differentiation by Bcl-2. Cancer Research 56:1199-1203, 1996.

12. Hartsell, TL, Yalowich, JC, Ritke, M, Martinez, AJ, Schor, NF. Induction of apoptosis in murine and human neuroblastoma cell lines by the enediyne natural product neocarzinostatin. J Pharm Exp Therap 275:479-485, 1995.

13. Schor, NF. Targeted enhancement of the biological activity of the antineoplastic agent, neocarzinostatin: Studies in murine neuroblastoma cells. J Clin Invest 89:774-781, 1992.

14. Beerman, TA, Poon, R, and Goldberg, IH. Single-strand nicking of DNA in vitro by neocarzinostatin and its possible relationship to the mechanism of drug action. Biochim. Biophys. Acta 475: 294-306, 1977.

15. DeGraff, WG, Mitchell, JB. Glutathione dependence of neocarzinostatin cytotoxicity and mutagenicity in Chinese hamster V-79 cells. Cancer Research 45:4760-4762, 1985.

16. McKelvey, EM, Murphy, W, Zander, A, Bodey, GP. Neocarzinostatin: report of a phase II clinical trial. Cancer Treat Reports 65:699-701, 1981.

17. Nicolaou, KC, Dai, W-M, Tsay, S-C, Estevez, VA, Wrasidlo, W. Designed enediynes: a new class of DNA-cleaving molecules with potent and selective anticancer activity. Science 256:1172-1178, 1992.

18. Maeda, H, Ueda, M, Morinaga, T, Matsumoto, T. Conjugation of poly(styrene-co-maleic acid) derivatives to the antitumor protein of neocarzinostatin: pronounced improvements in pharmacological properties. J Med Chem 28:455-461, 1985.

19. Hinman, LM, Hamann, PR, Wallace, R, Menendez, AT, Durr, FE, Upslacis, J. Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. Cancer Research 53:3336-3342, 1993.

20. Johnston, SR, MacLennan, KA, Sacks, NP, Salter, J, Smith, IE, Dowsett, M. Modulation of Bcl-2 and Ki-67 expression in oestrogen receptor-positive human breast cancer by tamoxifen. Eur J Cancer 30A:1663-1669, 1994.

21. Falcione, M, Milligan, KD, Schwartz, MC, Schor, NF. Prevention of neocarzinostatin-induced cell death and morphologic change in SK-N-SH human neuroblastoma cells by continuous exposure to nerve growth factor. Biochem Pharm 46:731-738, 1993.

22. Hartsell, TL, Hinman, LM, Hamann, PR, Schor, NF. Determinants of the response of neuroblastoma cells to DNA damage: the roles of pre-treatment cell morphology and chemical nature of the damage. J Pharm Exp Therap 277:1158-1166, 1996.

23. Cortazzo, MH, Kassis, ES, Sproul, KA, Schor, NF. p75/NGF-mediated protection of neuroblastoma cells from antimitotic agent-induced apoptosis. J. Neurosci. 16:3895-3899, 1996.

24. Schor NFT. Adjunctive use of Ethiofos (WR2721) with free radical-generating chemotherapeutic agents in mice: New caveats for therapy. Cancer Research 47:5411-5414, 1987.

25. Schor NF. Depletion of glutathione by the radioprotective agent, S-2-(3-aminopropylamino) ethyl phosphorothioic acid (WR2721). Biochem Pharm 37:562-563, 1988a.

26. Schor NF. Mechanisms of synergistic toxicity of the radioprotective agent, WR2721, and 6-hydroxydopamine. Biochem Pharm 37:1751-1762, 1988b.

27. Schor NF, Siuda JF, Lomis TJ, Cheng, B. Structural studies on the inactivation of γ–glutamylcysteine synthetase by the disulfide analogues of radioprotective cysteamine derivatives: Effects of aminoalkyl- and hydroxyalkyl- chain length and ß,ß-bis-dimethylation. Biochem J 267:291-296, 1990.

28. Schor, NF, Cheng, B, Siuda, JF. Inactivation of γ–glutamylcysteine synthetase by SAPH-3 disulfide: The role of the histaminyl moiety. J Pharm Sci 80:311-312, 1991.

29. Tyurina, YY, Tyurin, VA, Yalowich, JC, Quinn, PJ, Claycamp, HG, Schor, NF, Pitt, BR, Kagan, VE. Phenoxyl radicals of etoposide (VP-16) can directly oxidize intracellular thiols: Protective or damaging effects of phenolic antioxidants? Toxicol Appl Pharmacol 131: 277-288, 1995.

30. Boise, LH, Gonzalez-Garcia, M, Postema, CE, Ding, L, Lindsten, T, Turka, LA, Mao, X, Nunez, G, and Thompson, CB. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597-608, 1993.

31. Tietze, F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Analyt Biochem 27:502-522, 1969.

33. Schor NF. Neocarzinostatin induces neuronal morphology of mouse neuroblastoma in culture. J Pharm Exp Therap 249:906-910, 1989.

35. Ritov, VB, Banni, S, Yalowich, JC, Day, BW, Claycamp, HG, Corongiu, FP, Kagan, VE. Non-random peroxidation of different classes of membrane phospholipids in live cells detected by metabolically integrated *cis*-parinaric acid. Biochim Biophys Acta 1283:127-140, 1996.

32. Tyurina, Y.Y., Tyurin, V.A., Carta, G., Quinn, P.J., Schor, N.F., Kagan, V.E. Direct evidence for antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cells. Arch Biochem Biophys 344:413-423, 1997.

34. Schor, NF, Rudin, CM, Hartmann, A-R, Thompson, CB, Tyurina, YY, Kagan, VE. Bcl-2-induced alteration of glutathione handling and its exploitation in targeted cancer chemotherapy. Molec. Pharmacol, submitted, 1999.

36. Fabisiak, J.P., Kagan, V.E., Ritov, V.B., Johnson, D.E., Lazo, J.S. Bcl-2 inhibits selective oxidation and externalization of phosphatidylserine during paraquat-induced apoptosis. Am J Physiol 272 (Cell Physiol 41):C675-84, 1997.

37. Schor, N.F., Tyurina, Y.Y., Fabisiak, J.P., Tyurin, V.A., Lazo, L.S., Kagan, V.E. Selective oxidation and externalization of membrane phosphatidylserine: bcl-2-induced potentiation of the final common pathway for apoptosis. Brain Research, in press, 1999.

38. Schor, NF, Tyurina, YY, Tyurin, VA, Kagan, VE. Differential membrane antioxidant effects fo immediate and long-term estradiol treatment of MCF-7 breast cancer cells. Biochem. Biophys. Research Commun., in press, 1999.

39. Green, P.S., Gridley, K.E., Simpkins, J.W. Nuclear estrogen receptor-independent neuroprotection by estratrienes: a novel interaction with glutathione. Neuroscience 84:7-10, 1998.

40. Cai, J., Yang, J., Jones, D.P. Mitochondrial control of apoptosis: the role of cytochrome c. Biochim Biophys Acta 1366:139-49, 1998.

41. Cai, J., Jones, D.P. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. J Biol Chem 273:11401-4, 1998.

42. Han, Z., Bhalla, K., Pantazis, P., Hendrickson, E.A., Wyche, J.H. Cif (Cytochrome c efflux-inducing factor) activity is regulated by Bcl-2 and caspases an correlates with the activation of Bid. Mol Cell Biol 19:1381-9, 1999.

43. Lacort, M., Leal, A.M., Liza, M., Martin, C., Martinez, R., Ruiz-Larrea, M.B. Protective effect of estrogens and catecholestrogens against peroxidative membrane damage in vitro. Lipids 30:141-6, 1995.

44. Steinman, H. M. The Bcl-2 oncoprotein functions as a pro-oxidant. J Biol Chem 270:3487-3490, 1995.

45. Jacobson, M. D. and Raff, M. C. Programmed cell death and Bcl-2 protection in very low oxygen. Nature 374:814-816, 1995.

46. Halliwell, B., and Gutteridge, J. M. C. Oxygen radicals and the nervous system. TINS 8:22-26, 1985.

47. Dole, M., Nunez, G., Merchant, A. K., Maybaum, J., Rode, C. K., Bloch, C. A., and Castle, V. P. Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. Cancer Research 54:3253-3259, 1994.

48. Dole, M. G., Jasty, R., Cooper, M. J., Thompson, C. B., Nunez, G., and Castle, V. P. Bcl-x_L is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. Cancer Research 55:2576-2582,1995.

49. Beham, A. W., and McDonnell, T. J. Bcl-2 confers resistance to androgen deprivation in prostate carcinoma cells. Proc Amer Assoc Cancer Research 37:224, 1996.

50. Campos, L., Rouault, J.-P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J.-P., and Guyotat, D. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood 81:3091-3096, 1993.

51. Reber, U., Wullner, U., Trepel, M., Baumgart, J., Seyfried, J., Klockgether, T., Dichgans, J., and Weller, M. Potentiation of treosufan toxicity by the glutathione-depleting agent buthionine sulfoximine in human malignant glioma cells: the role of Bcl-2. Biochem Pharmacol 55:349-359, 1998.

52. Maeda, H. (1994) The clinical effects of neocarzinostatin and its polymer conjugate, SMANCS. In: Enediyne Antibiotics as Antitumor Agents Marcel Decker, NY, NY, pp.363-381.

53. Stoyanovsky, DA, Goldman, R, Jonnalagadda, SS, Day, BW, Claycamp, HG, Kagan, VE. Detection and characterization of the electron paramagnetic resonance-silent glutathionyl-5,5-dimethyl-1-pyrroline N-oxide adduct derived from redox cycling of phenoxyl radicals in model systems and HL-60 cells. Arch Biochem Biophys 330:3-11, 1996.

54. Chin, D.-H., Zeng, C.-H., Costello, C. E., and Goldberg, I. H. Sites in the diyne-ene bicyclic core of neocarzinostatin chromophore responsible for hydrogen abstraction from DNA. Biochemistry 27:8106-8114, 1988.

(11) APPENDICES

FIGURE LEGENDS

Figure 1. Western blot for human Bcl-2 Performed on Estradiol (E2) -deprived (10 d) MCF-7 Cells After 14 d Replacement with E2 (0 - $5x10^{-7}$ M). Western blotting was performed. A whole cell homogenate of 10^6 cells was applied to each lane. (A) Photograph of representative lanes of the Western blot depicting *bcl-2*-transfected PC12 cells as a positive control and MCF-7 cells initially estrogen-deprived and subsequently treated with $5x10^{-7}$ M E2. The numbers to the left of the gel photograph indicate the running position on the gel of various molecular weight standards. (B) Densitometric vertical scans of each lane of the Western blot stained for Bcl-2.

Figure 2. Effects of bcl-2 transfection on accumulation of GSH in tumor cells continuously incubated with N-acetylcysteine (NAcCys; 10 mM). The results at t = 0 represent the native GSH content of each of these cell lines. (A) PC12 pheochromocytoma cels. Four independent experiments were performed and gave comparable results. (B) MCF-7 breast cancer cells. The results of single determinations from each of two mock- and two bcl-2-transfected clones of MCF-7 cells are shown. A second independent experiment gave comparable results.

Figure 3. Effect of Immediate and Long-term Exposure to E2 and Treatment with AMVN on Glutathione Content of MCF-7 Breast Cancer Cells. Control, immediate, and long-term E2-exposed MCF-7 cells were incubated in the presence or absence of AMVN (500 μM) for 2 h at 37°C. After incubation, cells were washed twice with PBS, and reduced glutathione was determined by ThioGlo™ assay. Results shown are means of triplicate determinations and error bars signify SEM. Results for long-term E2-exposed cells treated in the absence of AMVN differ significantly from those for the corresponding control and immediate E2-exposed cells with $p < 0.0001$ (Student's t test). However, treatment with AMVN did not alter the glutathione content of either immediate or long-term E-2-exposed cells.

Figure 4. Effects of bcl-2 transfection on sensitivity of cultured tumor cells to NCS. In all cases, cells were treated for 1 h with NCS on day 0 and counted daily. Points represent the mean of counts from three separate high power fields expressed as a percent of the count on day 0. Error bars signify the SEM. (A) PC12 pheochromocytoma cells; (B) MCF-7 breast cancer cells.

Figure 5. Effect of Treatment with Estradiol (E2) on Oxidation of *cis*-Parinaric Acid (PnA)-labeled Phospholipids Induced by AMVN in MCF-7 Breast Cancer Cells. MCF-7 cells were treated with E2 (0 - $5x10^{-7}$ M) concurrently with (immediate; A) or for 14 days preceding (long-term; B) a 2 h AMVN treatment (500 μM; 37°C). Immediately prior to AMVN treatment, cells were loaded with PnA. Immediate E2 treatment afforded statistically significant protection against oxidation of PI and PS ($p < 0.02$ relative to AMVN alone at all E2 concentrations; Student's t test), and a trend towards protection against oxidation of PE at all E2 concentrations. In contrast, long-term E2 treatment

afforded statistically significant protection against oxidation of PS alone ($p < 0.03$ relative to AMVN alone at 10^{-8} and $5x10^{-7}$ M E2; Student's t test).

Figure 6. Effect of N-acetylcysteine (10 mM; 4 h) pretreatment on NCS sensitivity of PC12 pheochromocytoma eclls (A) and MCF-7 cells (B). NCS treatment (1 h) was performed on sister cultures immediately after washout of N-acetylcysteine (NAcCys) or vehicle (no NAcCys) from the medium. Results shown represent the mean cell counts of three determinations from one of two independent and comparable experiments. SEMs of the triplicate determinations are plotted but in some cases are too small to be resolved on the plot. Adherent cell counts are shown during mid-log phase growth (day 5 for PC12 cells and day 3 for MCF-7 cells).

Figure 1A

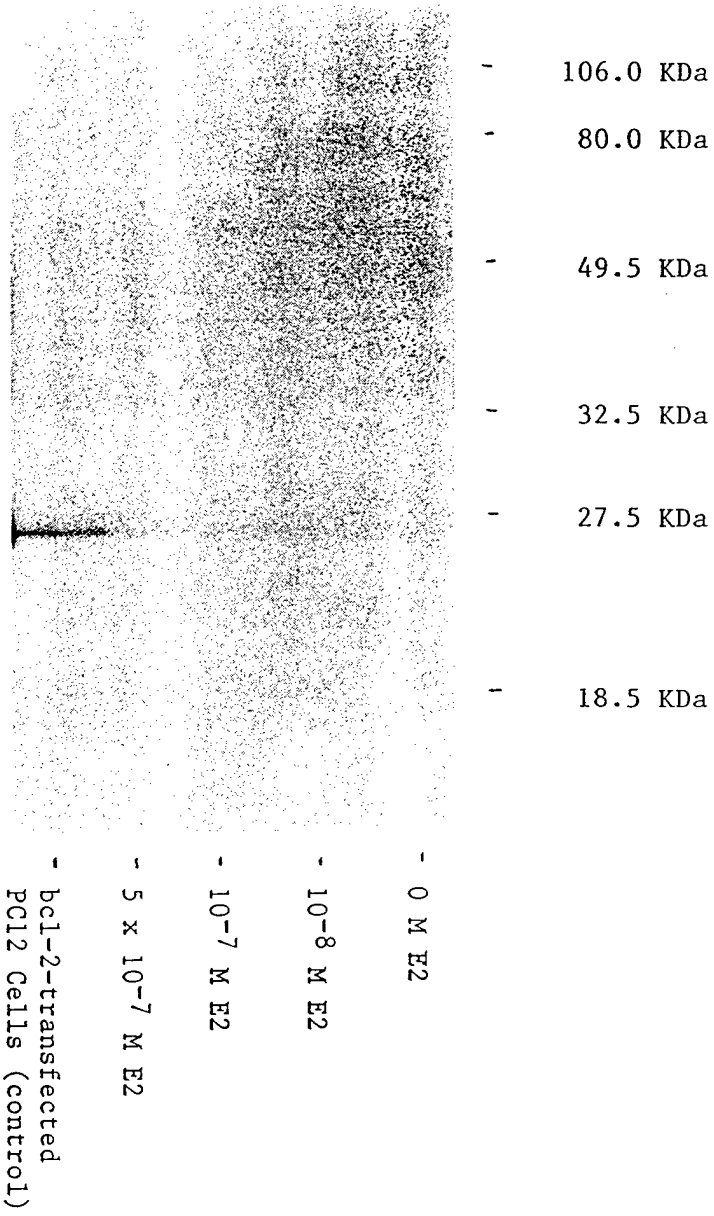

106.0 KDa
80.0 KDa
49.5 KDa
32.5 KDa
27.5 KDa
18.5 KDa
- 0 M E2
- 10^{-8} M E2
- 10^{-7} M E2
- 5 x 10^{-7} M E2
- bcl-2-transfected PC12 Cells (control)

Figure 1B

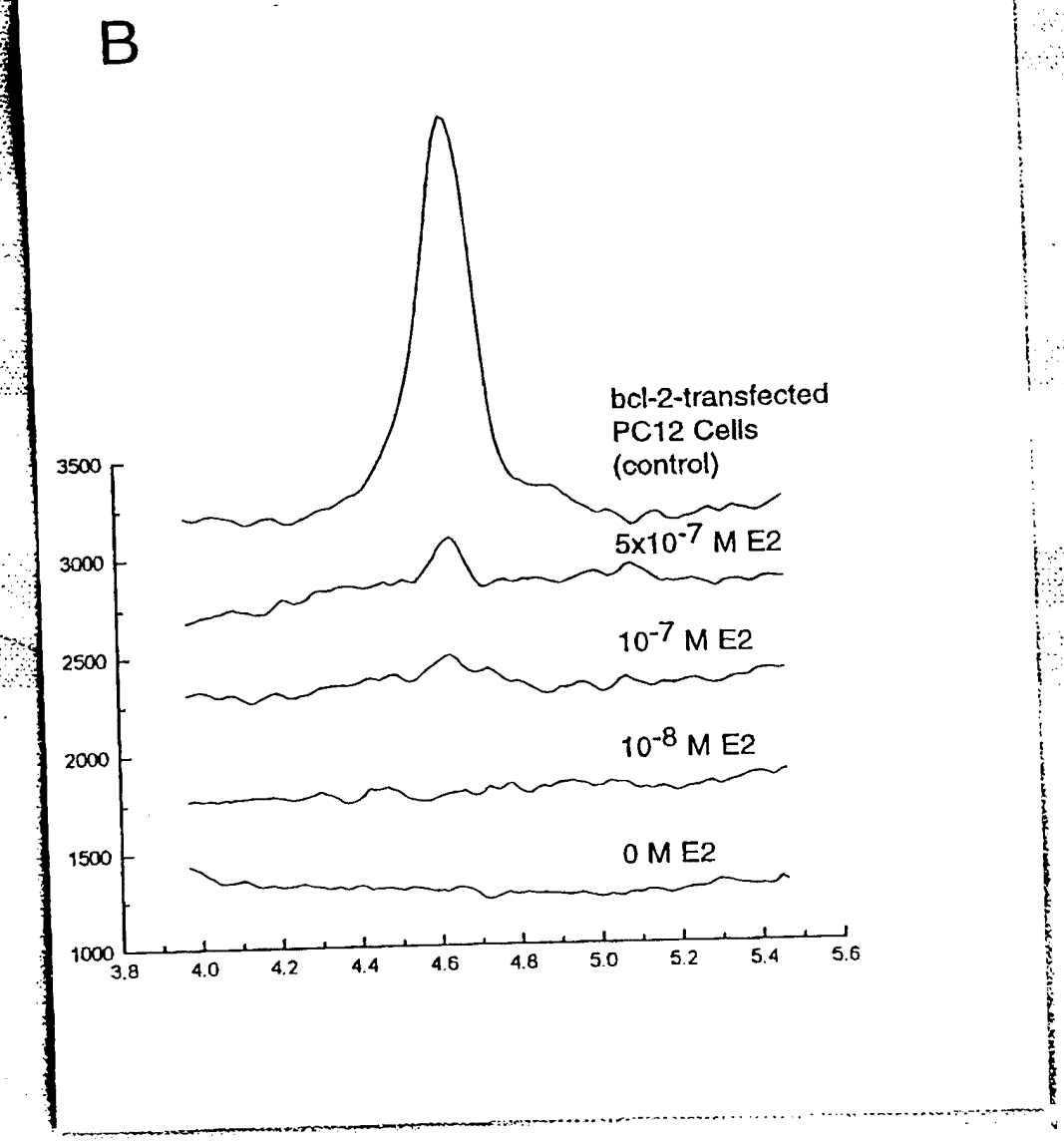

B
bcl-2-transfected
PC12 Cells
(control)
5x10-7 M E2
10-7 M E2
10-8 M E2
0 M E2
3500
3000
2500
2000
1500
1000
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6

Figure 2A

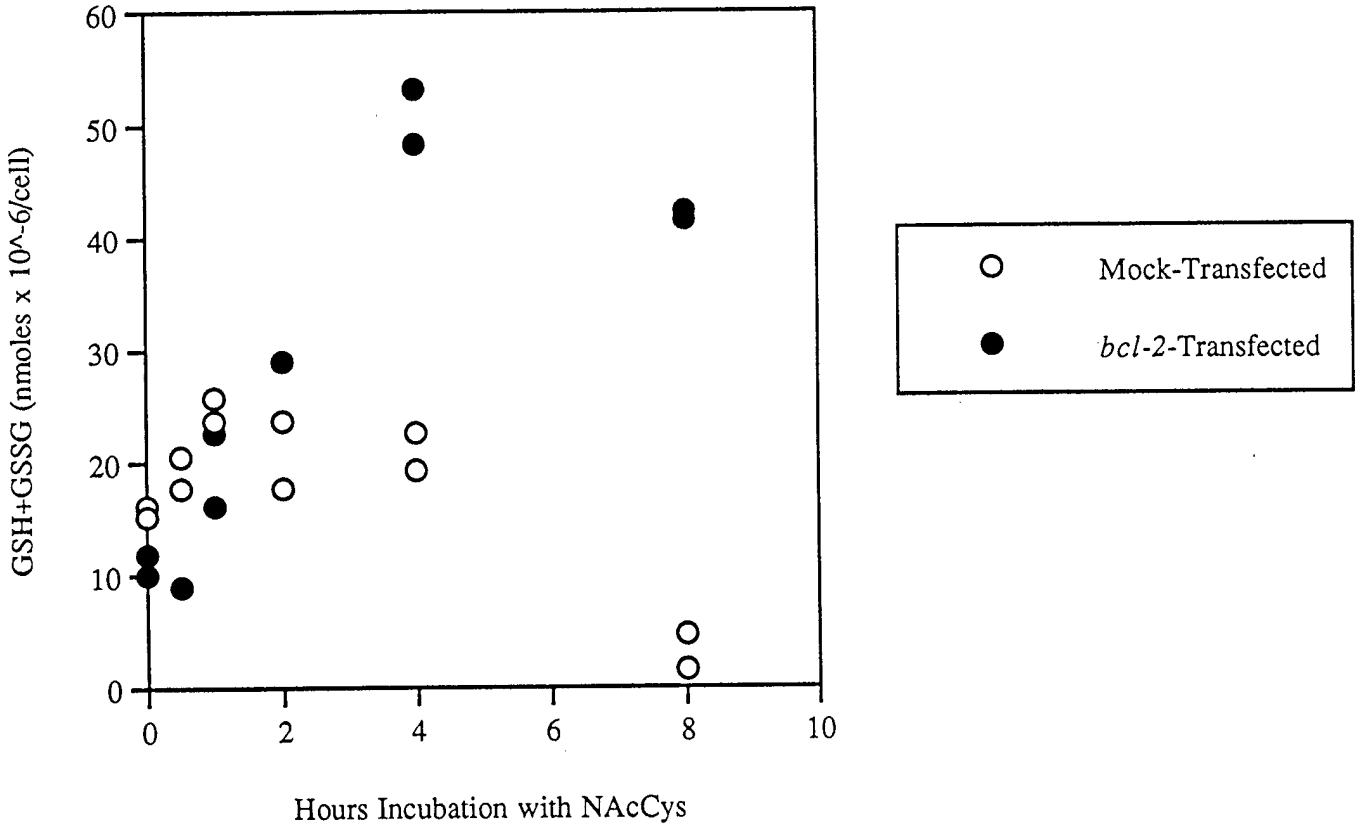

GSH+GSSG (nmoles x 10^-6/cell)
60
50
40
30
20
10
0
0
2
4
6
8
10
Hours Incubation with NAcCys
Mock-Transfected
bcl-2-Transfected

Figure 2B

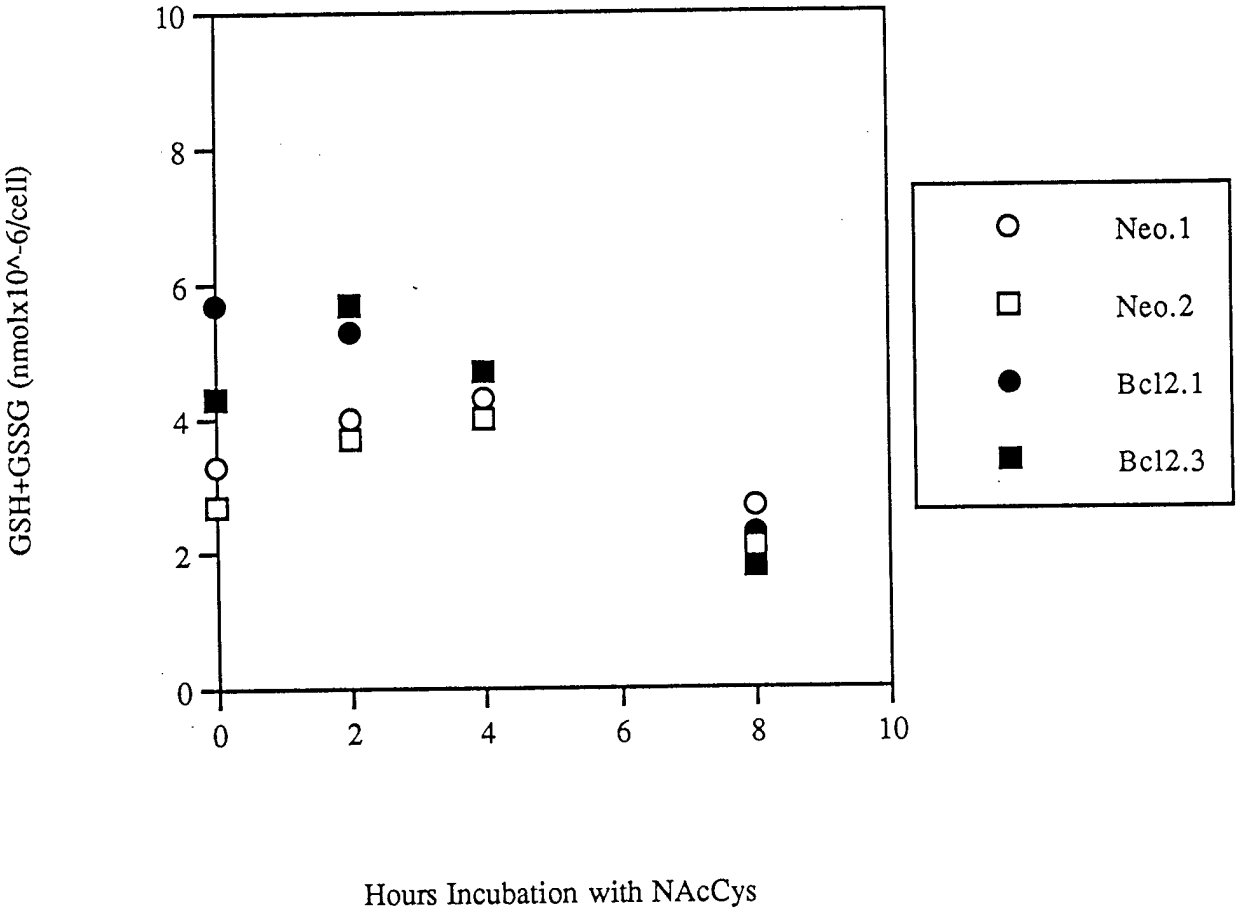
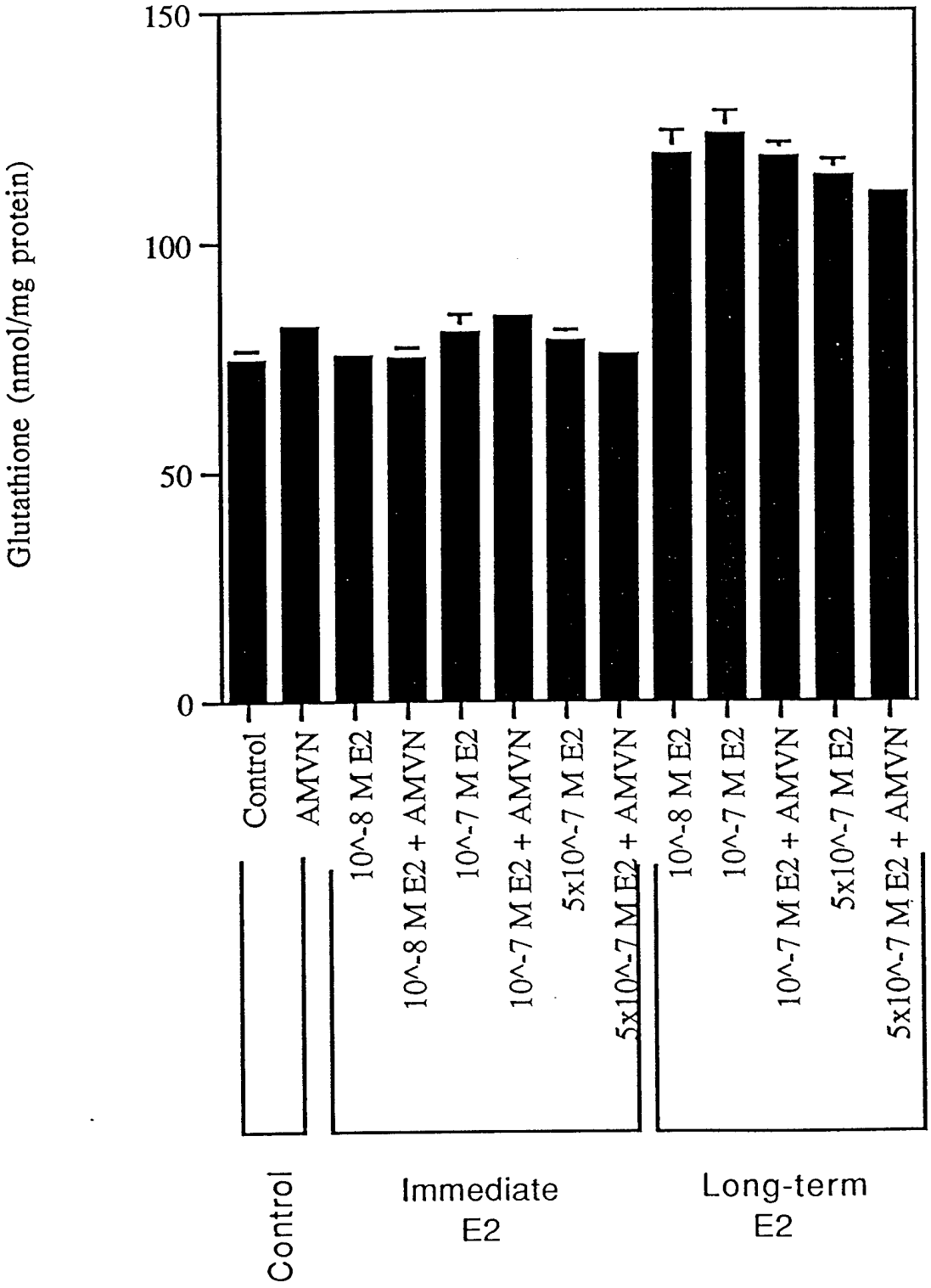

GSH+GSSG (nmolx10^-6/cell)
10
8
6
4
2
0
0
2
4
6
8
10
Hours Incubation with NAcCys
Neo.1
Neo.2
Bcl2.1
Bcl2.3

Figure 3


Glutathione (nmol/mg protein)
150
100
50
0
Control
AMVN
10^-8 M E2
10^-8 M E2 + AMVN
10^-7 M E2
10^-7 M E2 + AMVN
5x10^-7 M E2
5x10^-7 M E2 + AMVN
10^-8 M E2
10^-7 M E2
10^-7 M E2 + AMVN
5x10^-7 M E2
5x10^-7 M E2 + AMVN
Control
Immediate E2
Long-term E2

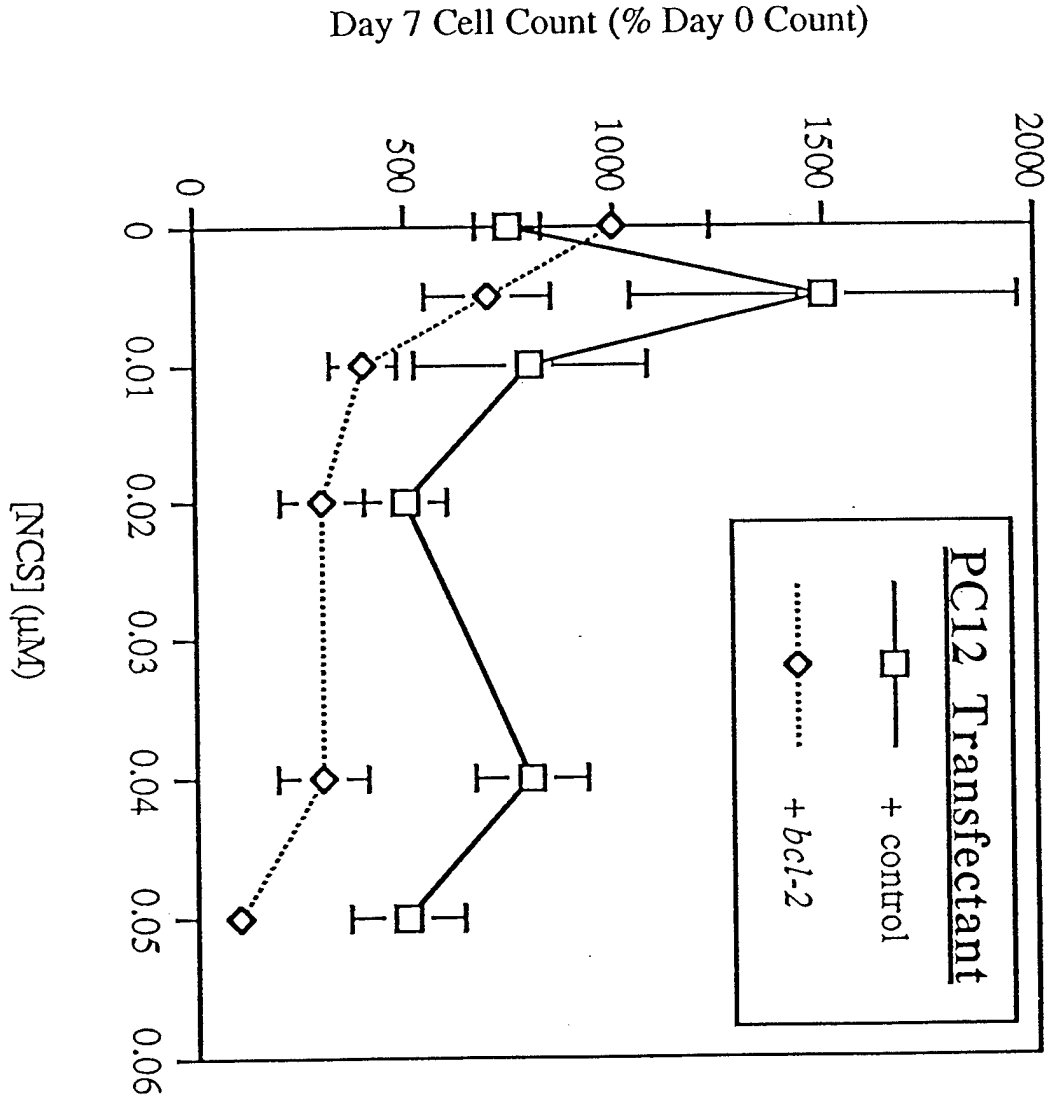
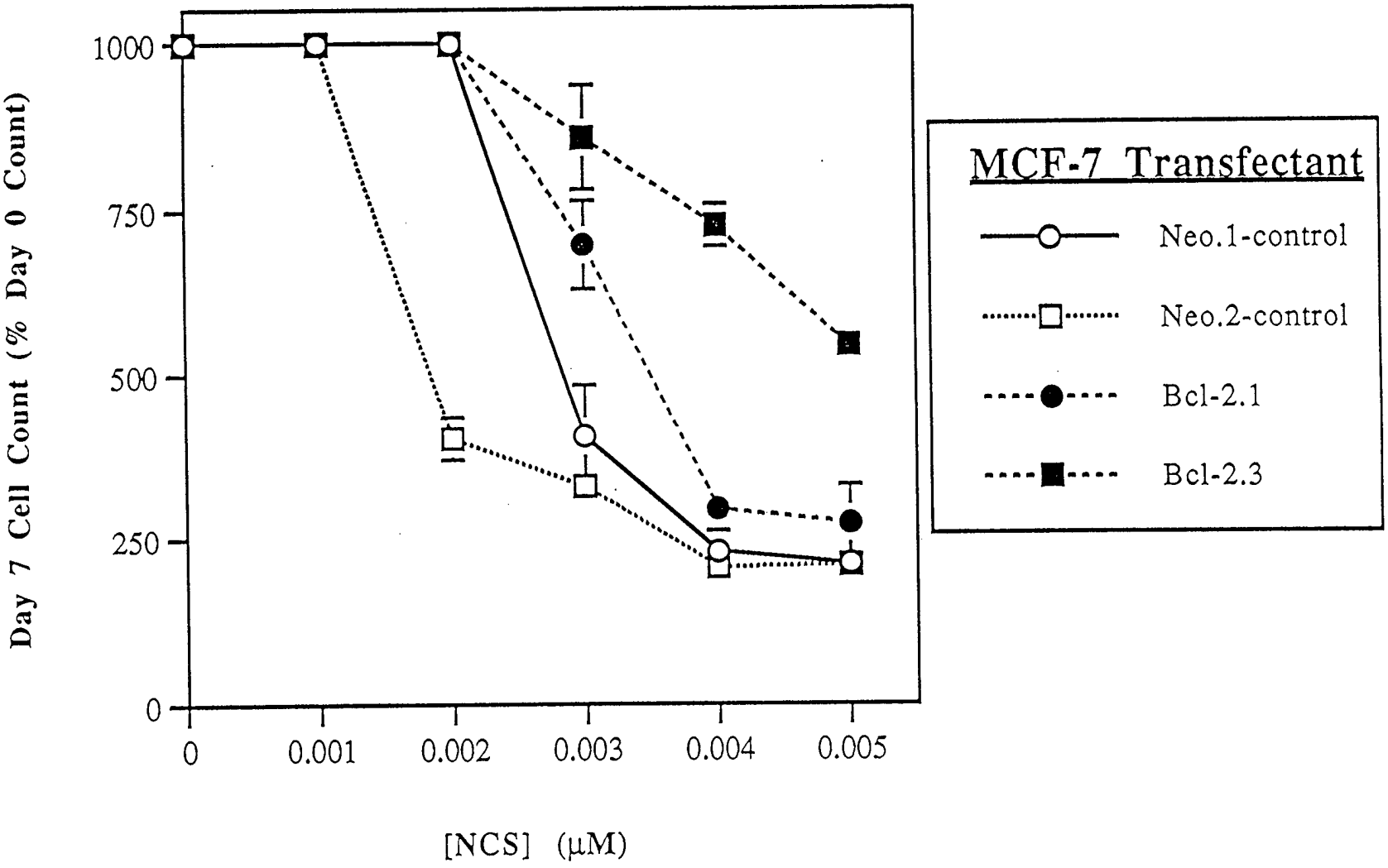

Day 7 Cell Count (% Day 0 Count)
2000
1500
1000
500
0
0
0.01
0.02
0.03
0.04
0.05
0.06
[NCS] (μM)
PC12 Transfectant
+ control
+ bcl-2

Figure 4A

Figure 4B


Day 7 Cell Count (% Day 0 Count)
1000
750
500
250
0
0
0.001
0.002
0.003
0.004
0.005
[NCS] (μM)
MCF-7 Transfectant
Neo.1-control
Neo.2-control
Bcl-2.1
Bcl-2.3

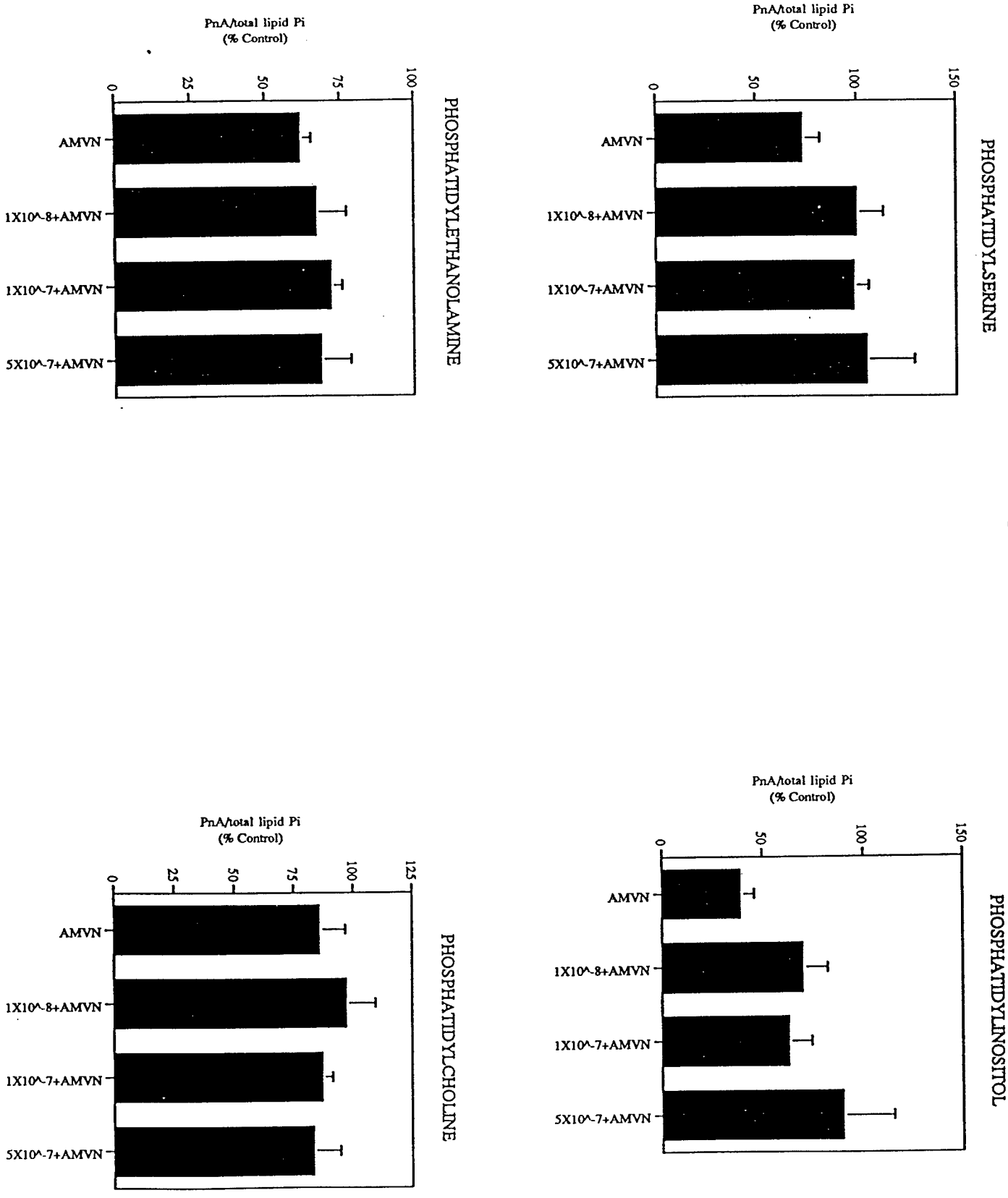
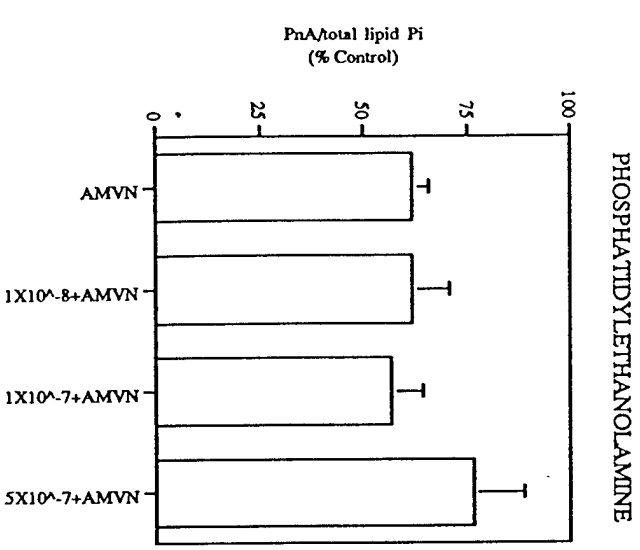

PnA/total lipid Pi
(% Control)
0
50
100
150
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLSERINE
PnA/total lipid Pi
(% Control)
0
25
50
75
100
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLETHANOLAMINE
PnA/total lipid Pi
(% Control)
0
50
100
150
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLINOSITOL
PnA/total lipid Pi
(% Control)
0
25
50
75
100
125
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLCHOLINE

Figure 5A


PnA/total lipid Pi
(% Control)
0
25
50
75
100
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1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLETHANOLAMINE

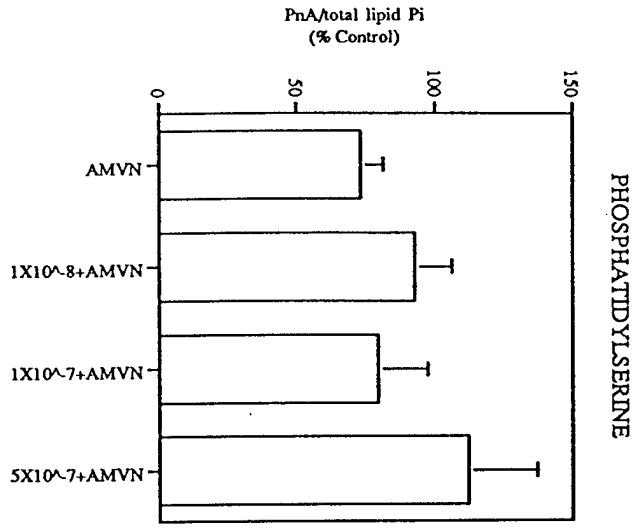
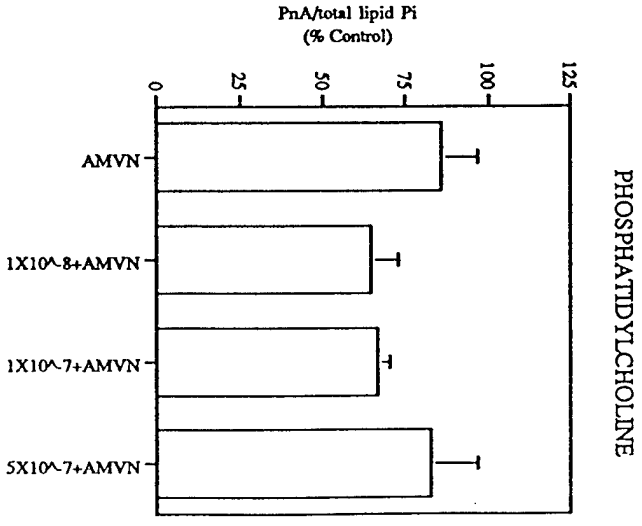
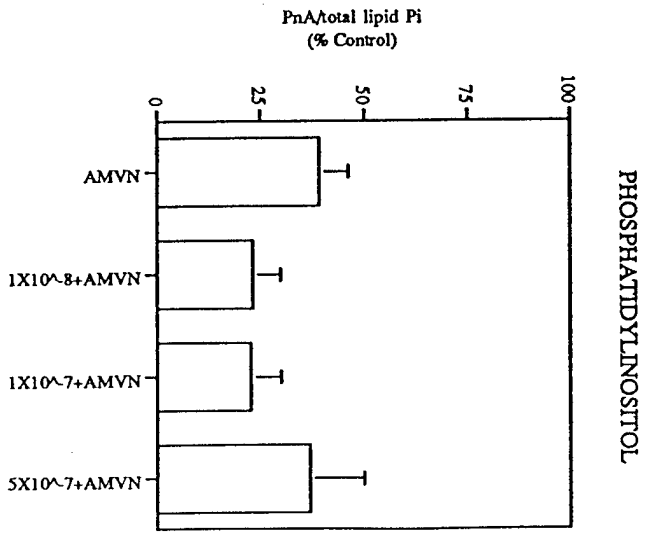

PnA/total lipid Pi
(% Control)
0
50
100
150
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLSERINE

Figure 5B


PnA/total lipid Pi
(% Control)
0
25
50
75
100
125
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLCHOLINE


PnA/total lipid Pi
(% Control)
0
25
50
75
100
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLINOSITOL

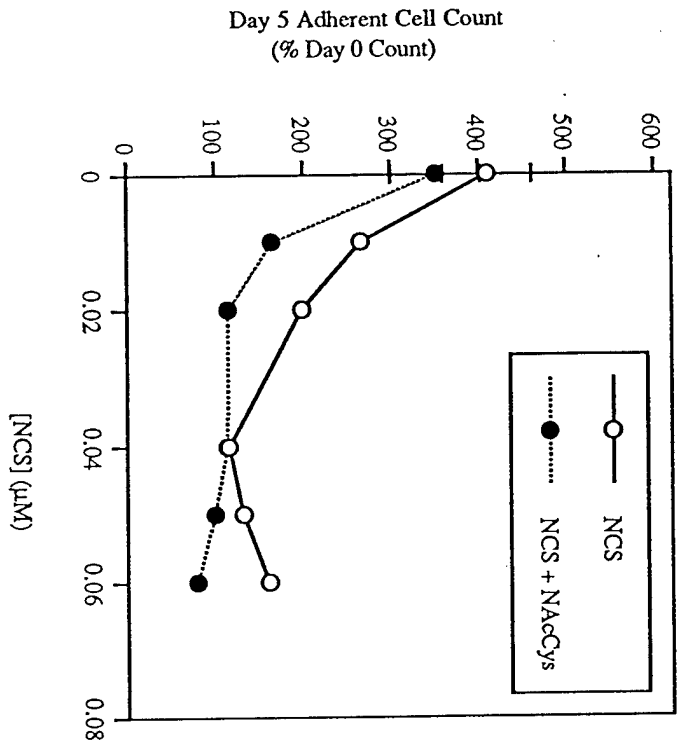

Day 5 Adherent Cell Count
(% Day 0 Count)
0
100
200
300
400
500
600
0
0.02
0.04
0.06
0.08
[NCS] (μM)
NCS
NCS + NAcCys

Figure 6A

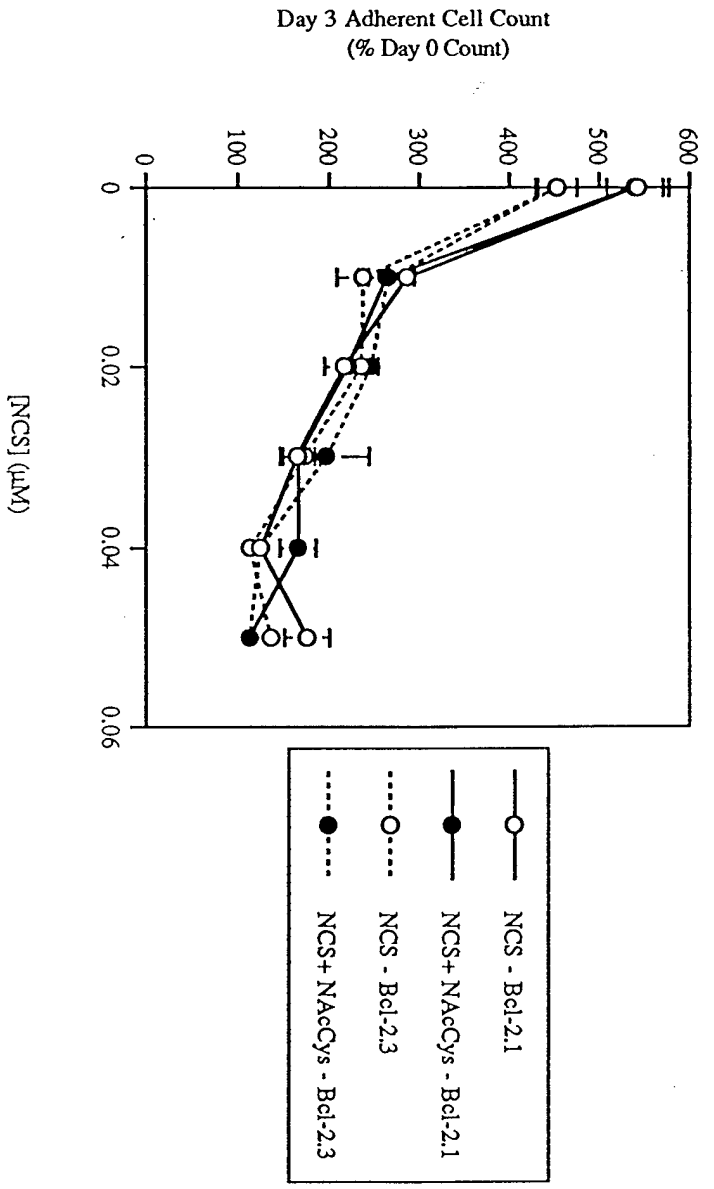

Day 3 Adherent Cell Count
(% Day 0 Count)
600
500
400
300
200
100
0
0
0.02
0.04
0.06
[NCS] (μM)
NCS - Bcl-2.1
NCS+ NAcCys - Bcl-2.1
NCS - Bcl-2.3
NCS+ NAcCys - Bcl-2.3

Figure 6B

TABLES

TABLE 1: Specific Incorporation of *cis*-Parinaric Acid (PnA) Into Membrane Phospholipids of MCF-7 Breast Cancer Cells. MCF-7 cells were membrane-loaded with PnA and the steady-state specific incorporation of PnA into the various membrane phospholipids was determined.

Phospholipids	Specific Incorporation (mol PnA : mol phospholipid)
Phosphatidylcholine	1 : 4
Phosphatidylethanolamine	1 : 10
Phosphatidylserine	1 : 13
Phosphatidylinositol	1 : 31

TABLE 2: Effect of AMVN on Membrane Phospholipids as Reflected by Fluorescence-detected Levels of *cis*-Parinaric Acid (PnA). MCF-7 cells were membrane-loaded with PnA and treated for 2 h (37°C) with vehicle or an equivalent volume of AMVN (500 μM). Peroxidation of PnA in the various membrane phospholipids was determined.

	ngPnA/mg Pi			
	PI	**PE**	**PS**	**PC**
Control (n=13)	35.5±14.9	242.3±25.6	37.7±6.5	1105.4±117.6
AMVN (n-5)	13.9±2.5	149.4±9.5	27.6±3.1	948.6±120.5
"p" vs. Control	0.006	< 0.001	0.005	0.023

TABLE 3: Effect of AMVN on Membrane Phospholipid Composition of MCF-7 Breast Cancer Cells. MCF-7 cells were incubated in phenol red-free DMEM in the absence or presence of AMVN (500 μM; 2 h; 37°C). Phospholipid composition was determined by HPTLC. All data are expressed as mean ± SEM, and n = 6 for each value. *$p < 0.05$ vs. control cells (Student's t test).

Phospholipid	Percent of Total Phospholipids	
	Control	AMVN
Diphosphatidylglycerol	2.33 ± 0.48	3.07 ± 0.41
Phosphatidylethanolamine	27.85 ± 0.28	25.61 ± 0.92
Phosphatidylcholine	47.62 ± 0.24	48.94 ± 0.88
Phosphatidylserine	5.63 ± 0.15	5.76 ± 0.33
Phosphatidylinositol	7.89 ± 0.27	6.90 ± 0.37
Sphingomyelin	8.33 ± 0.03	8.14 ± 0.30
Lysophosphatidylcholine	0.48 ± 0.11	1.14 ± 0.25*

Brain Research, In Press

17 Manuscript Pages (including Figures)

3 Figures; 0 Tables

SELECTIVE OXIDATION AND EXTERNALIZATION OF MEMBRANE PHOSPHATIDYLSERINE: Bcl-2-INDUCED POTENTIATION OF THE FINAL COMMON PATHWAY FOR APOPTOSIS

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ABSTRACT

The induction of apoptosis in PC12 cells by the enediyne neocarzinostatin (NCS) is paradoxically potentiated by overexpression of *bcl-2*. The enhanced activation of NCS seen in *bcl-2*-overexpressing cells cannot by itself be responsible for the potentiation of apoptosis, since Bcl-2 would be expected to block apoptosis at a point distal to NCS activation (e.g., in the apoptosis final common pathway). We now report that overexpression of *bcl-2* in PC12 cells does not protect the cells from NCS-induced oxidation of membrane phosphatidylserine (PS), and results in potentiation of NCS-induced externalization of membrane PS, two events associated with the apoptosis final common pathway. The mechanism of potentiation of apoptosis by Bcl-2 is related to the enhanced reducing potential of *bcl-2*-overexpressing PC12 cells.

CLASSIFICATION TERMS

Theme: Development and Regeneration

Topic: Neuronal Death

Key Words: apoptosis; membrane peroxidation; phospholipids; phosphatidylserine

INTRODUCTION

The induction of apoptosis in neural cells has been hypothesized to play a role in the morphological and physiological changes associated with a variety of developmental and pathological states [7]. For many of these, reactive oxygen species (ROS) have been implicated in the disease pathogenesis itself and/or as final common mediators of apoptosis [18]. Similarly, species known to influence the induction and enactment of apoptosis have been proposed as modulators of development and disease, and cellular handling of ROS. Studies of the effects of *bcl-2* overexpression on ROS handling in neural cells have suggested a role for Bcl-2 in increasing the tolerance of such cells to oxidant stress [1,5,15]. Similarly, there is mounting evidence that the resistance of neural crest tumor cells to chemotherapeutic agent-induced apoptosis is related to the expression by these cells of *bcl-2* [3,4]. The mechanism by which these phenomena occur is not entirely clear, but some studies have indicated that the cellular content of reducing species is increased in neural cells that overexpress *bcl-2* [5]. We have previously demonstrated that apoptosis induced in neural crest tumor cells by the enediyne antineoplastic prodrug, neocarzinostatin (NCS), is potentiated by overexpression of *bcl-2* [2]. We hypothesized that this paradoxical potentiation of apoptosis is related to increased reductive activation of NCS in *bcl-2* overexpressing cells. Other studies have suggested, however, that the antioxidant and anti-apoptosis activities of Bcl-2 are related to its action downstream of the initial activity of chemotherapeutic agents and other apoptosis-inciting factors [9,19]. This would suggest that, even if a chemotherapeutic prodrug were activated to a greater extent in *bcl-2*-overexpressing cells, Bcl-2 should theoretically block the apoptotic pathway distal to that inciting event (i.e., at a point in the final common apoptosis pathway). We have therefore attempted to resolve this seeming paradox by examining the effects of NCS on changes associated specifically with the common apoptosis pathway in mock- and *bcl-2*-transfected

PC12 cells. Previous studies in our laboratory have demonstrated the role of peroxidation and externalization of membrane-bound phosphatidylserine (PS) early in the enactment phase of the apoptosis pathway [6]. They have further shown the activity of Bcl-2 in preventing peroxidation of PS and other membrane phospholipids by paraquat or the azo-initiator of lipid peroxyl radicals, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN; [6,15]). We now report the effects of Bcl-2 on membrane phospholipid peroxidation and PS externalization in PC12 cells treated with NCS.

MATERIALS AND METHODS

Cell lines and transfectants: Mock- and *bcl-2*-transfected PC12 cells, both selected as polyclonal populations [5], were the kind gift of Dr. Dale E. Bredesen (Burnham Institute, La Jolla, CA). These cells were maintained as adherent monolayers as we have previously described [2].

Incorporation of parinaric acid (PnA) into membrane phospholipids: Membrane phospholipids of both transfectants were labeled with PnA as we have described previously [15]. Briefly, mock- and *bcl-2*-transfected PC12 cells were incubated with a PnA-human serum albumin complex for 2 h at 37°C in L1210 medium. After incubation, lipids were extracted and resolved by HPLC.

Fluorescent labeling and analysis of external membrane phospholipids: Derivitization of intact cells with fluorescamine was performed as we have previously described [12]. Two-dimensional HPTLC was then performed on a total lipid extract of 10^6 logarithmically-growing cells using chloroform:methanol:28% ammonium hydroxide::65:25:5 in the first dimension and chloroform:acetone:methanol:glacial acetic acid:water::50:20:10:10:5 in the second dimension.

Visualization of externalized PS: Because of the specific propensity of annexin V to bind to PS only on the extracellular face of the cell membrane, this compound has been used to "stain" cells for the presence of this translocated PS [6,10,11,14,16,17]. Dual staining of mock- and *bcl-2*-transfected cells with propidium iodide and fluorescein-labeled annexin V [6] was performed at 24 h after completion of a 1 h exposure to NCS.

Dose-response studies of enediynes: Dose-response studies of the enediynes NCS and C1027 in apoptosis induction in mock- and *bcl-2*-transfected PC12 cells were performed as we have described previously [2]. In each case and for each drug, the ED_{50} for apoptosis induction was defined and determined as detailed by Hartsell et al. [8].

RESULTS

Membrane lipid peroxidation by NCS in mock- and *bcl-2*-transfected PC12 cells: The membranes of mock- and *bcl-2*-transfected PC12 cells were saturated with PnA as a probe for membrane phospholipid oxidation [12,15]. The time course and pattern of incorporation of PnA into membrane phospholipids did not differ significantly between mock- and *bcl-2*-transfected PC12 cells (data not shown). Similar to the case for AMVN treatment, a 1 h exposure of mock-transfected PC12 cells to NCS (37°C; 1-5 $\mu g/10^6$ cells) resulted in statistically significant (20-30%) peroxidation of phosphatidylinositol (PI), phosphatidylethanolamine (PEA), phosphatidylcholine (PC), and PS (Fig. 1). However, unlike the case for AMVN treatment, overproduction of Bcl-2 did not protect PC12 cells from PS oxidation induced by exposure to NCS. The selectivity of this phenomenon is demonstrated by the statistically significant protection by Bcl-2 of all other membrane phospholipids studied from NCS-induced peroxidation.

Externalization of PS in mock- and *bcl-2*-transfected PC12 cells treated with NCS: Immediately after a 1 h exposure to NCS, both PEA and PS are externalized in an NCS concentration-dependent fashion, as determined by direct fluorescamine staining of external membrane phospholipids (Fig. 2). Externalization of PS is approximately 2-fold greater in *bcl-2*-transfected than in mock-transfected PC12 cells ($p < 0.01$). In contrast, externalization of PEA did not differ significantly between the transfectants at any NCS concentration.

Neither transfectant exposed to control conditions demonstrated staining with annexin V (Figs. 3a,d), indicating that membrane PS normally faces intracellularly in these cells. Treatment with NCS resulted in *bcl-2*-transfected cells that stained with annexin V, even at 0.5 μg/ml, the lowest NCS concentration examined (Figs. 2e,f). In contrast, no mock-transfected cells stained with annexin V at 0.5 μg/ml NCS (Fig. 3b), and exposure to 2.5 μg/ml NCS resulted scant staining of an occasional cell (Fig. 3c). These studies suggest that, unlike the case for treatment with AMVN or paraquat, Bcl-2 does not abrogate, but rather potentiates, PS oxidation and externalization in PC12 cells.

Role of reductive activation in potentiation of NCS-induced apoptosis by Bcl-2: From a mechanistic standpoint, potentiation by Bcl-2 of NCS-induced apoptosis (ED_{50}: PC12, 0.02 μM; PC12/Bcl-2, 0.005 μM) is dependent on the requirement for reductive activation of this prodrug. Mock- and *bcl-2*-transfected PC12 cells treated with the reduction-independent, autoactivating enediyne, C1027, demonstrated *bcl-2*-induced protection from, rather than potentiation of apoptosis (ED_{50}: PC12, $1x10^{-5}$ μM; PC12/Bcl-2, $2x10^{-5}$ μM).

DISCUSSION

Recent studies have demonstrated the protective effect of Bcl-2 against apoptosis produced by direct triggering of the final common apoptosis pathway [13,20]. Our previous observation that Bcl-2 overproduction potentiates the induction of apoptosis in PC12 cells by NCS would appear to contradict this notion. If indeed Bcl-2 acts only at a distal common point in the induction of apoptosis, then NCS-induced apoptosis should be abrogated by Bcl-2, even if Bcl-2 directly or indirectly enhances the activation of NCS. The present studies demonstrate potentiation by Bcl-2 of events in the final common apoptosis pathway.

In T lymphocytes, initiation of apoptosis is accompanied by down-regulation of the ATP-dependent amino-phospholipid translocase and activation of a nonspecific phospholipid scramblase [6,10]. Both of these changes result in transfer of PS from the inner to the outer surface of the cell membrane. The effects of Bcl-2 on PS externalization induced by NCS was studied both by direct fluorescamine staining of external membrane phospholipids and annexin V staining of external PS in intact cells. The results of these studies indicate that potentiation by Bcl-2 of NCS-induced apoptosis is accompanied by selective maintenance of vulnerability to oxidation of PS and potentiation of early events in the common pathway for apoptosis.

The potentiation of NCS-induced apoptosis has been hypothesized to be related to increased activation of this enediyne in the highly reducing intracellular milieu of *bcl-2*-transfected cells [2,5]. The present studies comparing autoactivating and reduction-requiring enediynes definitively demonstrate the role of reductive activation in this model. While it is possible that NCS-induced apoptosis is not susceptible to the regulatory influence of Bcl-2, it is perhaps more likely that increased activation of NCS results in consumption of species critical to the anti-apoptosis effects of Bcl-2, or in the quantitative overriding of the protective activity of Bcl-2. In its most specific application, the

potentiation, by overexpression of *bcl-2* in PC12 cells, of NCS activity and PS oxidation and externalization suggests that *in vitro* screening for such potentiation could be used to identify chemoresistant tumors that might be responsive to treatment with enediynes. In a more general sense, this potentiation raises the caveat that, in neurodegenerative disorders for which gene therapy with *bcl-2* or its relatives has been suggested, such therapy must be carefully assessed in light of other aspects of the pharmacological and physiological environment in which the cells in question sit.

REFERENCES

1. H. Albrecht, J. Tschopp, C.V. Jongeneel, Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-kappa B by TNF, *FEBS Lett* 351 (1994) 45-48.

2. M. Cortazzo, N.F. Schor, Potentiation of enediyne-induced apoptosis and differentiation by Bcl-2, *Cancer Res* 56 (1996) 1199-1203.

3. M. Dole, G. Nunez, A.K. Merchant, J. Maybaum, C.K. Rode, C.A. Bloch, V.P. Castle, Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma, *Cancer Res* 54 (1994) 3253-3259.

4. M.G. Dole, R. Jasty, M.J. Cooper, C.B. Thompson, G. Nunez, V.P., Bcl-X_L is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis, *Cancer Res* 55 (1995) 2576-2582.

5. L.M. Ellerby, H.M. Ellerby, S.M. Park, A.L. Holleran, G. Fiskum, D.J. Kane, M.P. Testa, C. Kayalar, D.E. Bredesen, Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2, *J Neurochem* 67 (1996) 1259-1267.

6. J.P. Fabisiak, V.E. Kagan, V.B. Ritov, D.E. Johnson, J.S. Lazo, Bcl-2 inhibits selective oxidation and externalization of phosphatidylserine during paraquat-induced apoptosis, *Am J Physiol* 272 Cell Physiol 41 (1997) C675-C684.

7. H.A. Gelbard, R.M. Boustany, N.F. Schor, Apoptosis in development and disease of the nervous system, Part 2: Apoptosis in childhood neuologic disease, *Pediatr Neurol* 16 (1997) 93-97.

8. T.L. Hartsell, L.M. Hinman, P.R. Hamann, N.F. Schor, Determinants of the response of neuroblastoma cells to DNA damage: The roles of pretreatment cell morphology and chemical nature of the damage, *J Pharm Exp Therap* 277 (1996) 1158-1166.

9. R.M. Kluck, E. Bossy-Wetzel, D.R. Green, D.D. Newmeyer, The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis, *Science* 275 (1997) 1132-1136.

10. G. Koopman, C.P. Reutelingsperger, G.A. Kuijten, R.M. Keehnen, S.T. Pals, M.H. van Oers, Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis, *Blood* 84 (1995) 1415-1420.

11. C. Ravanat, G. Archipoff, A. Beretz, G. Freund, J.P. Cazenave, J.M. Freyssinet, Use of annexin-V to demonstrate the role of phosphatidylserine exposure in the maintenance of haemostatic balance by endothelial cells, *Biochem J* 282 Pt. 1 (1992) 7-13.

12. V.B. Ritov, S. Banni, J.C. Yalowich, B.W. Day, H.G. Claycamp, F.P. Corongiu, V.E. Kagan, Non-random peroxidation of different classes of membrane phospholipids in live cells detected by metabolically integrated cis-parinaric acid, *Biochim Biophys Acta* 1283 (1996) 127-140.

13. T. Rosse, R. Olivier, L. Monney, M. Rager, S. Conus, I. Fellay, B. Jansen, C. Borner, Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c, *Nature* 391 (1998) 496-499.

14. J.F. Tait, D. Gibson, Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content, *Arch Biochem Biophys* 298 (1992) 187-191.

15. Y.Y. Tyurina, V.A. Tyurin, G. Carta, P.J. Quinn, N.F. Schor, V.E. Kagan, Direct evidence for antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cells, *Arch Biochem Biophys* 344 (1997) 413-423.

16. B. Verhoven, R.A. Schlegel, P. Williamson, Rapid loss and restoration of lipid asymmetry by different pathways in resealed erythrocyte ghosts, *Biochim Biophys Acta* 1026 (1990) 153-160.

17. I. Vermes, C. Haanen, H. Steffens-Nakken, C. Reutelingsperger, Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, *J Immunol Methods* 184 (1995) 39-51.

18. K.A. Wood, R.J. Youle, Apoptosis and free radicals, *Ann NY Acad Sci* 738 (1994) 400-407.

19. J. Yang, X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.-I. Peng, D.P. Jones, X. Wang, Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked, *Science* 275 (1997) 1129-1132.

20. B. Zhivotovsky, S. Orrenius, O.T. Brustugun, S.O. Doskeland, Injected cytochrome c induces apoptosis, *Nature* 391 (1998) 449-450.

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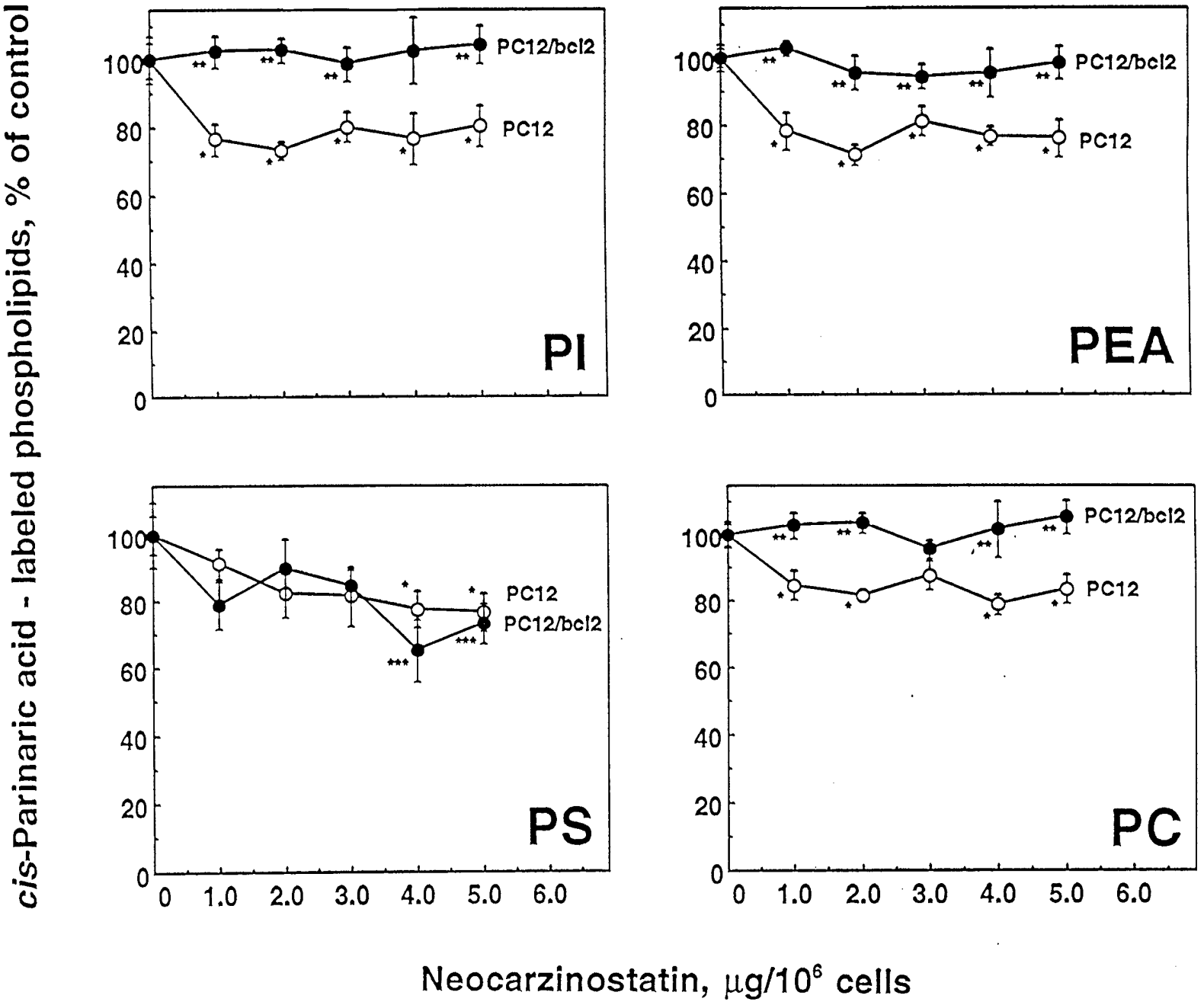
FIGURE LEGENDS

Fig. 1. Neocarzinostatin-induced oxidation of *cis*-parinaric acid-labeled [12,15] phospholipids in mock- and *bcl-2*-tranfected PC12 cells. Each point represents the mean ± SEM of five determinations made in two independent experiements. Abbreviations: PnA, *cis*-parinaric acid; NCS, neocarzinostatin; PC12, mock-transfected PC12 cells; PC12/bcl-2, *bcl-2*-transfected PC12 cells; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. Student's t test analysis: *$p < 0.05$ vs. vehicle-treated PC12 cells; **$p < 0.05$ vs. NCS-treated PC12 cells; ***$p < 0.05$ vs. vehicle-treated PC12/bcl-2 cells.

Fig. 2. Neocarzinostatin- (NCS-) induced externalization of phosphatidylethanolamine (PEA; panel A) and phosphatidylserine (PS; panel B). Mock- and *bcl-2*-transfected PC12 cells were incubated with NCS (0 - 4 μg/10^6 cells) for 1 h at 37°C in the dark in L1210 medium. The cells were then rinsed twice and external phospholipids were derivatized with fluorescamine. The total cell lipids were then extracted and analyzed by two-dimensional HPTLC [12]. Open bars, mock-transfected cells; hatched bars, *bcl-2*-transfected cells. Data shown represent the mean ± SEM of 5 determinations in 3 independent experiments. *$p < 0.05$ vs. vehicle-treated cells; **$p < 0.001$ vs. vehicle-treated cells; $^{\#}p < 0.01$ vs. *bcl-2*-transfected cells treated with the same concentration of NCS.

Fig. 3. Representative fluorescence micrographs of control and NCS-treated mock- and *bcl-2*-transfected PC12 cells dual-stained with propidium iodide and fluorescein conjugated annexin V [6]. PC12 cells transfected with *bcl-2* (d-f) and mock-transfected PC12 cells (a-c) following 24 h exposure to 0.5 μg/ml (0.05 μM; b,e) or 2.5 μg/ml (0.25 μM; c,f) NCS. Vehicle-treated cells are shown in panels a and d. Each of the panels shown is representative of 5 different high-power fields (400 X).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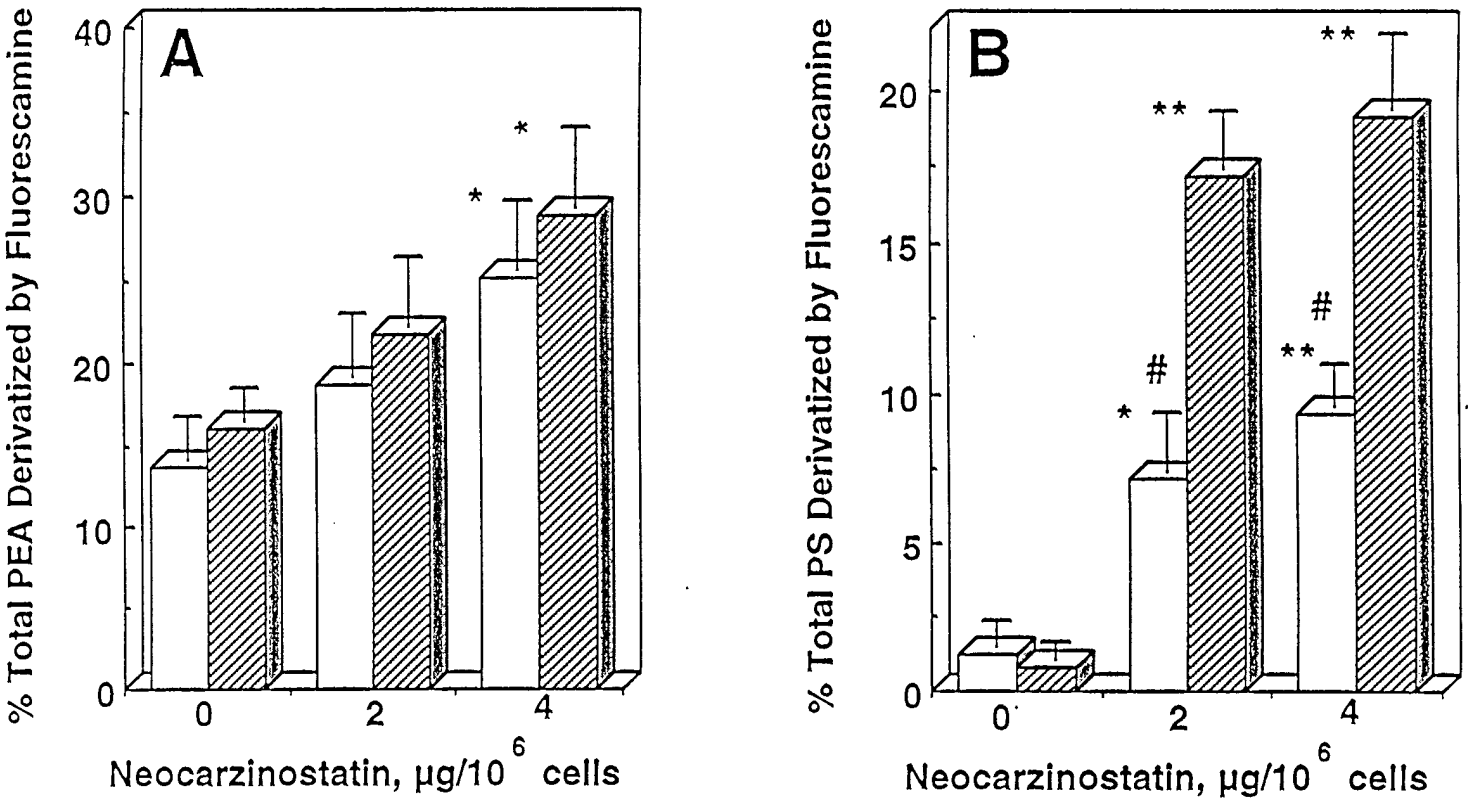
PC12
PI
PEA
PS
PC
0
20
40
60
80
100
0
1.0
2.0
3.0
4.0
5.0
6.0
Neocarzinostatin, μg/10⁶ cells

Figure 1

Figure 2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% Total PEA Derivatized by Fluorescamine
40
30
20
10
0
0
2
4
Neocarzinostatin, µg/10 6 cells
*
*
B
% Total PS Derivatized by Fluorescamine
20
15
10
5
0
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4
Neocarzinostatin, µg/10 6 cells
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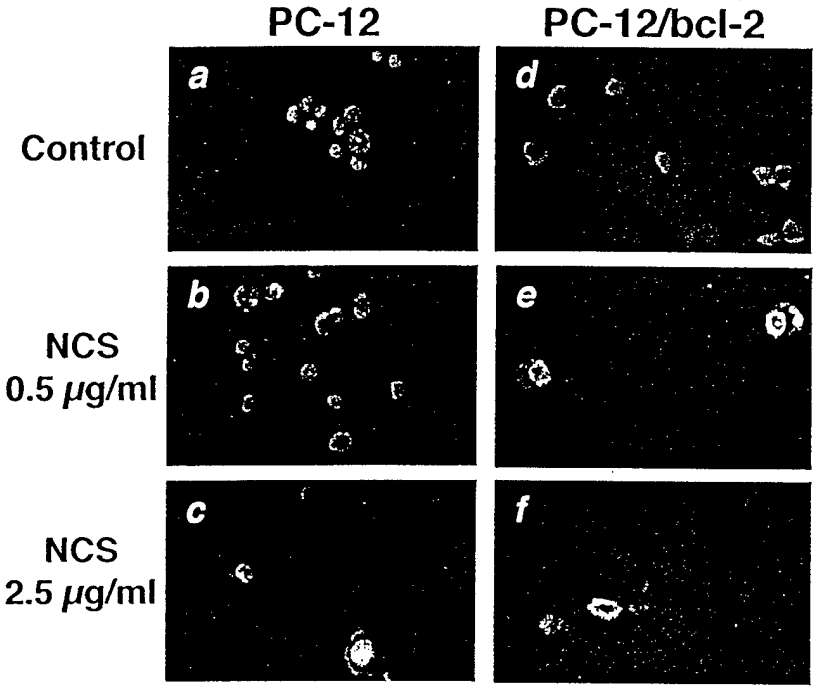
Figure 3



Biochem, Biophys Res. Commun, In Press

DIFFERENTIAL MEMBRANE ANTIOXIDANT EFFECTS OF IMMEDIATE AND LONG-TERM ESTRADIOL TREATMENT OF MCF-7 BREAST CANCER CELLS

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ABSTRACT

Previous studies have documented the direct antioxidant effects of estradiol, and it is tempting to ascribe the antiapoptosis effects of estradiol to its scavenging of reactive oxygen species. However, recent reports have also demonstrated that long-term exposure of MCF-7 human breast cancer cells to estradiol results in estrogen receptor- and estradiol dose-dependent overexpression of the antiapoptosis gene, *bcl-2*. We have used the pattern of protection of membrane phospholipids from oxidation as a probe to separate these direct and indirect effects of estradiol from one another. Immediate exposure to estradiol non-specifically protects all membrane phospholipids from oxidation by the diazo radical initiator, AMVN. This implies the direct antioxidant activity of estradiol in this system. In contrast, long-term exposure, with associated increased expression of *bcl-2*, protects only phosphatidylserine, the oxidation of which is a critical component of the final common pathway for apoptosis. This *bcl-2*-mediated indirect effect of estradiol is accompanied by prevention of apoptosis in MCF-7 cells.

Direct, estrogen receptor-independent antioxidant effects have been identified for estrogen in a number of systems (1-3). Synergy with glutathione has been demonstrated in some, leading to the proposal of combined estrogen-antioxidant therapy for neurodegenerative disorders thought to have their origins in the generation of reactive oxygen species (4). This direct effect of estrogen is not exposure time-dependent; that is, it is observed immediately upon addition of estrogen to the system.

Similarly, the antiapoptosis protein, Bcl-2, has been hypothesized to play direct (5) and indirect (6) roles in altering the redox state of the cell. Studies demonstrating an estradiol concentration- and estrogen receptor-dependent induction of the *bcl-2* gene in MCF-7 human breast cancer cells (7) have led us to predict that estradiol would also have an indirect antioxidant and antiapoptotic effect on these cells. Unlike its direct effects, the induction of *bcl-2* by estradiol requires long-term (i.e., days to weeks) estradiol exposure.

Our previous studies have demonstrated that the diazo radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN; Polysciences, Inc., Warrington, PA) induces the concentration-dependent incidence of apoptosis in PC12 pheochromocytoma cells, and that Bcl-2 abrogates apoptosis induction and membrane phospholipid oxidation in these cells (8). While such oxidation in general may accompany or be etiologic in the induction of and commitment of a cell to apoptosis, the specific oxidation and translocation of phosphatidylserine appear to accompany the enactment phase of the apoptotic process (9,10). The pattern of oxidation of individual phospholipids can therefore suggest the point at which reactive oxygen species and their modification play a role in the incidence and/or abrogation of apoptosis. In order to test the hypothesis that the mechanistic relationship between the antioxidant and anti-apoptosis effects of estradiol depends on the duration of estradiol exposure, we have examined the pattern of oxidation of phospholipids in the membranes of MCF-7 cells by AMVN after the addition of estradiol to the medium immediately or for 14 days preceding AMVN treatment.

MATERIALS AND METHODS

MCF-7 cells (ATCC, Rockville, MD) were maintained as adherent monolayers in 75mm^2 tissue culture flasks (Life Technologies, Grand Island, NY) fed twice weekly with α-MEM (Mediatech, Herndon, VA) supplemented with 5% FBS (Atlanta Biologicals, Norcross, GA), 0.3% glucose, 2 mM L-glutamine (Life Technologies, Grand Island, NY), and 2 μg/ml gentamicin sulfate (Biofluids, Rockville, MD). In the case of immediate estradiol treatment, the medium was removed and cells were maintained for 24 days in estrogen-free, phenol-free DMEM (Biofluids) supplemented with 5% charcoal-treated FBS (Cocalico Biological, Inc., Reamstown, PA). Estradiol was then added at the time of AMVN treatment. In the case of long-term estradiol treatment, prior to each experiment, the medium was removed and replaced with estrogen-free, phenol-free DMEM. The cells were maintained in this estrogen-free medium for 10 days. Subsequently, this medium was replaced with estrogen-free medium supplemented with varying concentrations of 17β-estradiol (E2; 0 - $5x10^{-7}$ M; Sigma, St. Louis, MO), and the cells were maintained in the supplemented medium for an additional 14 days.

The membranes of the cells were loaded with *cis*-parinaric acid (PnA; Molecular Probes, Eugene, OR) as we have previously described (8), as an *in situ* probe for the peroxidation of membrane phospholipids. Upon peroxidation, PnA loses its fluorescence, making the fluorescence of each of the membrane phospholipids into which it has been incorporated a measure of the peroxidation of those phospholipids (11).

RESULTS AND DISCUSSION

As is shown in Fig. 1, long-term E2 supplementation resulted in a concentration-dependent increase in the Bcl-2 content of the cells. Immediate E2 supplementation did not affect cellular Bcl-2 content (data not shown).

Specific incorporation of PnA into membrane phospholipids of MCF-7 cells is shown in Table 1. PnA served as an *in situ* probe for phospholipid oxidation, as we have previously described (8,11). Treatment of control MCF-7 cells with AMVN (500 μM; 2 h at 37°C) resulted in statistically significant peroxidation of all four phospholipid species examined (Table 2). The fraction of membrane phospholipid peroxidized varied slightly from 3.8% [for phosphatidylethanolamine (PE)] to approximately 2% [for phosphatidylinositol (PI)] of the native total phospholipid content. Phosphatidylcholine (PC) and phosphatidylserine (PS) demonstrated intermediate degrees of peroxidation. This oxidation occurred without sufficient change in the overall phospholipid composition of the membranes to induce necrotic death of the cells (Table 3).

Immediate E2 exposure of MCF-7 cells resulted in statistically significant protection from AMVN-induced peroxidation of PI and PS, and a trend towards such protection of PE and PC (i.e., decreased mean peroxidized fraction after AMVN exposure relative to non-E2-exposed cells that did not reach statistical significance; Fig. 2A). This wholesale protection of all phospholipids studied from peroxidation suggests a direct antioxidant role for estrogen in this system.

On the other hand, long-term E2 exposure resulted in the statistically significant protection of PS and, at the highest E2 concentration only, PE (Fig. 2B). This pattern is suggestive of Bcl-2-mediated protection from enactment of apoptosis (9,10), rather than direct protection from membrane phospholipid peroxidation *per se*. Protection of PS in this paradigm was accompanied by a 2-fold decrease in the percentage of the cells demonstrating apoptotic morphology after a 24 h exposure to AMVN (500 μM). Our previous studies demonstrated that apoptotic morphology peaks at 24 h in AMVN-treated PC12 cells (8). Apoptosis was seen in 40 ± 4 (SEM) percent of estrogen-deprived cells exposed to AMVN, and 22 ± 2 percent of long-term E2-treated ($5x10^{-7}$ M) cells similarly exposed ($p < 0.01$, Student's t test).

In light of the previous reports of glutathione-dependent (4) and independent (12-14) effects of E2 and Bcl-2 on apoptosis, we examined the effect of immediate and long-term E2 exposure on the glutathione content of MCF-7 cells. We also determined the effect of AMVN on the glutathione content of immediate and long-term E2-exposed cells. As is shown in Figure 3, immediate E2 exposure had no effect on the glutathione content of MCF-7 cells, while long-term exposure resulted in an increase in glutathione levels. At the concentrations of E2 used, this increase was not concentration-dependent. Regardless of the length of E2 exposure, however, AMVN treatment did not result in a decrement in glutathione levels, indicating that despite the increase in glutathione content that accompanies long-term E2 exposure, the protective effects of such exposure are likely glutathione-independent. This is in concert with previous reports of increased glutathione content related to *bcl-2* overexpression, and the independence from glutathione of the effects of Bcl-2 on apoptosis (15).

These results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (4), but may be enhanced by induction of estrogen metabolism (1). In contrast, *bcl-2* induction associated with long-term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (7). The present studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechnisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.

REFERENCES

1. Lacort, M., Leal, A.M., Liza, M., Martin, C., Martinez, R., Ruiz-Larrea, M.B. (1995) *Lipids* **30**, 141-6.

2. Carr, D.B., Goate, A., Phil, D., Morris, J.C. (1997) *Am J Med* **103**, 35-105.

3. Koh, K.K., Bui, M.N., Wincemoyer, R., Cannon, R.O. 3rd. (1997) *Am J Cardiol* **80**, 1505-7.

4. Green, P.S., Gridley, K.E., Simpkins, J.W. (1998) *Neuroscience* **84**, 7-10.

5. Albrecht, H., Tschopp, J., Jongeneel, C.V. (1994) *FEBS Lett* **351**, 45-8.

6. Ellerby, L.M., Ellerby, H.M., Park, S.M., Holleran, A.L., Fiskum, G., Kane, D.J., Testa, M.P., Kayalar, C., Bredesen, D.E. (1996) *J Neurochem* **67**, 1259-67.

7. Teixeira, C., Reed, J.C., Pratt, M.A. (1995) *Cancer Research* **55**, 3902-7.

8. Tyurina, Y.Y., Tyurin, V.A., Carta, G., Quinn, P.J., Schor, N.F., Kagan, V.E. (1997) *Arch Biochem Biophys* **344**, 413-23.

9. Fabisiak, J.P., Kagan, V.E., Ritov, V.B., Johnson, D.E., Lazo, J.S. (1997) *Am J Physiol* **272** (Cell Physiol **41**), C675-84.

10. Schor, N.F., Tyurina, Y.Y., Fabisiak, J.P., Tyurin, V.A., Lazo, L.S., Kagan, V.E. (1999) *Brain Research*, in press.

11. Ritov, V.B., Banni, S., Yalowich, J.C., Day, B.W., Claycamp, H.G., Corongiu, F.P., Kagan, V.E. (1996) *Biochim Biophys Acta* **1283**, 127-40.

12. Cai, J., Yang, J., Jones, D.P. (1998) *Biochim Biophys Acta* **1366**, 139-49.

13. Cai, J., Jones, D.P. (1998) *J Biol Chem* **273**, 11401-4.

14. Han, Z., Bhalla, K., Pantazis, P., Hendrickson, E.A., Wyche, J.H. (1999) *Mol Cell Biol* **19**, 1381-9.

15. Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T., Bredesen, D.E. (1993) *Science* **262**, 1274-7.

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TABLE 1: Specific Incorporation of *cis*-Parinaric Acid (PnA) Into Membrane Phospholipids of MCF-7 Breast Cancer Cells. MCF-7 cells were membrane-loaded with PnA and the steady-state specific incorporation of PnA into the various membrane phospholipids was determined as we have previously described (11).

Phospholipids	Specific Incorporation (mol PnA : mol phospholipid)
Phosphatidylcholine	1 : 4
Phosphatidylethanolamine	1 : 10
Phosphatidylserine	1 : 13
Phosphatidylinositol	1 : 31

TABLE 2: Effect of AMVN on Membrane Phospholipids as Reflected by Fluorescence-detected Levels of *cis*-Parinaric Acid (PnA). MCF-7 cells were membrane-loaded with PnA (11) and treated for 2 h (37°C) with vehicle or an equivalent volume of AMVN (500 μM). Peroxidation of PnA in the various membrane phospholipids was determined as we have previously described (11).

	ngPnA/mg Pi			
	PI	**PE**	**PS**	**PC**
Control (n=13)	35.5±14.9	242.3±25.6	37.7±6.5	1105.4±117.6
AMVN (n-5)	13.9±2.5	149.4±9.5	27.6±3.1	948.6±120.5
"p" vs. Control	0.006	< 0.001	0.005	0.023

TABLE 3: Effect of AMVN on Membrane Phospholipid Composition of MCF-7 Breast Cancer Cells. MCF-7 cells were incubated in phenol red-free DMEM in the absence or presence of AMVN (500 μM; 2 h; 37°C). Phospholipid composition was determined by HPTLC as we have previously described (8). All data are expressed as mean ± SEM, and n = 6 for each value. *p < 0.05 vs. control cells (Student's t test).

	Percent of Total Phospholipids	
Phospholipid	Control	AMVN
Diphosphatidylglycerol	2.33 ± 0.48	± 0.41
Phosphatidylethanolamine	27.85 ± 0.28	25.61 ± 0.92
Phosphatidylcholine	47.62 ± 0.24	48.94 ± 0.88
Phosphatidylserine	5.63 ± 0.15	5.76 ± 0.33
Phosphatidylinositol	7.89 ± 0.27	6.90 ± 0.37
Sphingomyelin	8.33 ± 0.03	8.14 ± 0.30
Lysophosphatidylcholine	0.48 ± 0.11	1.14 ± 0.25*

FIGURE LEGENDS

Figure 1. Western blot for human Bcl-2 Performed on Estradiol (E2) -deprived (10 d) MCF-7 Cells After 14 d Replacement with E2 (0 - $5x10^{-7}$ M). Western blotting was performed as we have previously described (8). A whole cell homogenate of 10^6 cells was applied to each lane.

Figure 2. Effect of Treatment with Estradiol (E2) on Oxidation of *cis*-Parinaric Acid (PnA) -labeled Phospholipids Induced by AMVN in MCF-7 Breast Cancer Cells. MCF-7 cells were treated with E2 (0 - $5x10^{-7}$ M) concurrently with (immediate; A) or for 14 days preceding (long-term; B) a 2 h AMVN treatment (500 μM; 37°C). Immediately prior to AMVN treatment, cells were loaded with PnA as we have previously described (8,11). Immediate E2 treatment afforded statistically significant protection against oxidation of PI and PS ($p < 0.02$ relative to AMVN alone at all E2 concentrations; Student's t test), and a trend towards protection against oxidation of PE at all E2 concentrations. In contrast, long-term E2 treatment afforded statistically significant protection against oxidation of PS alone ($p < 0.03$ relative to AMVN alone at 10^{-8} and $5x10^{-7}$ M E2; Student's t test).

Figure 3. Effect of Immediate and Long-term Exposure to E2 and Treatment with AMVN on Glutathione Content of MCF-7 Breast Cancer Cells. Control, immediate, and long-term E2-exposed MCF-7 cells were incubated in the presence or absence of AMVN (500 μM) for 2 h at 37°C. After incubation, cells were washed twice with PBS, and reduced glutathione was determined by ThioGlo™ assay (8). Results shown are means of triplicate determinations and error bars signify SEM. Results for long-term E2-exposed cells treated in the absence of AMVN differ significantly from those for the corresponding control and immediate E2-exposed cells with $p < 0.0001$ (Student's t test). However, treatment with AMVN did not alter the glutathione content of either immediate or long-term E-2-exposed cells.

Figure 1

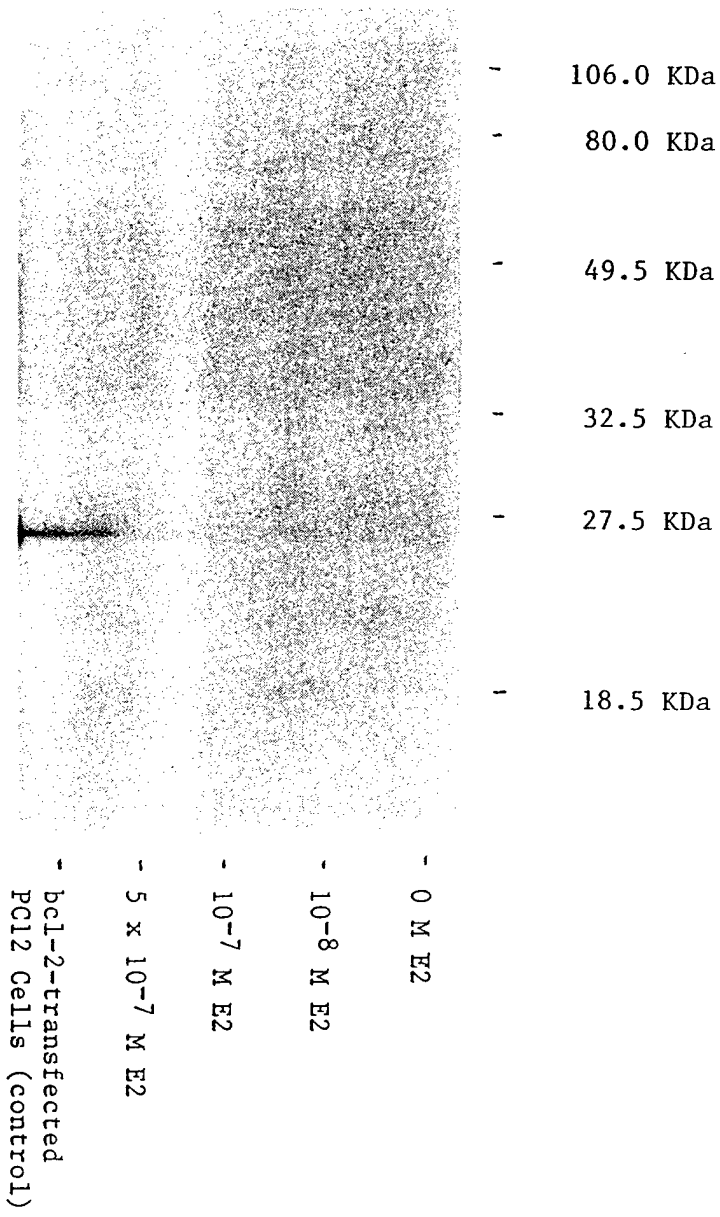
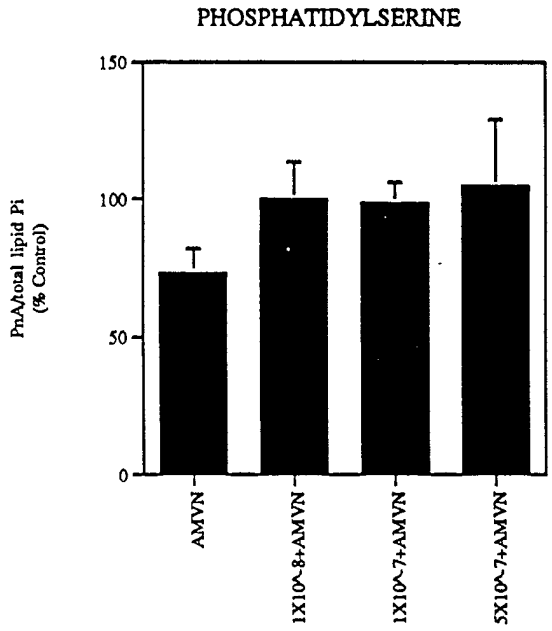
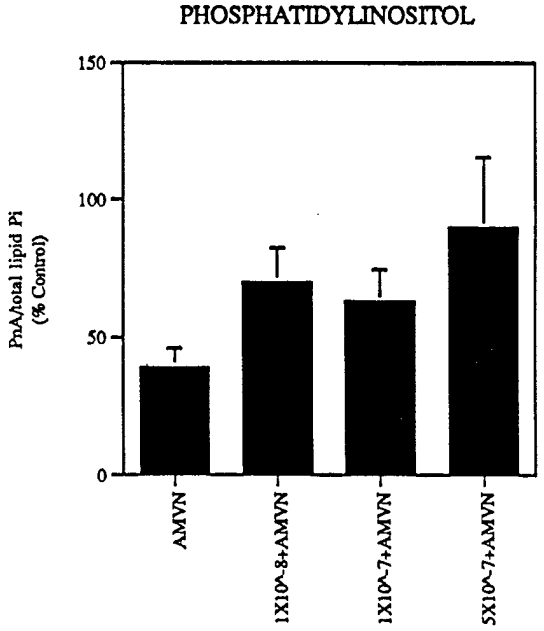
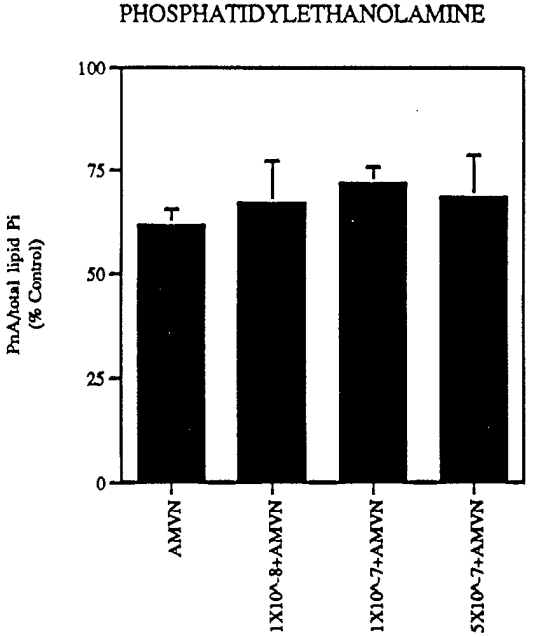

106.0 KDa
80.0 KDa
49.5 KDa
32.5 KDa
27.5 KDa
18.5 KDa
- 0 M E2
- 10^{-8} M E2
- 10^{-7} M E2
- 5 x 10^{-7} M E2
- bcl-2-transfected PC12 Cells (control)

Figure 2A


PHOSPHATIDYLSERINE
PnA/total lipid Pi
(% Control)
150
100
50
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN


PHOSPHATIDYLINOSITOL
PnA/total lipid Pi
(% Control)
150
100
50
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN


PHOSPHATIDYLETHANOLAMINE
PnA/total lipid Pi
(% Control)
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75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN

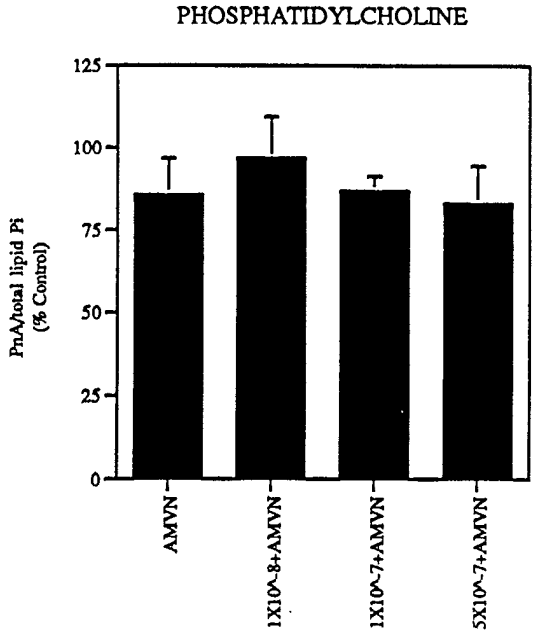
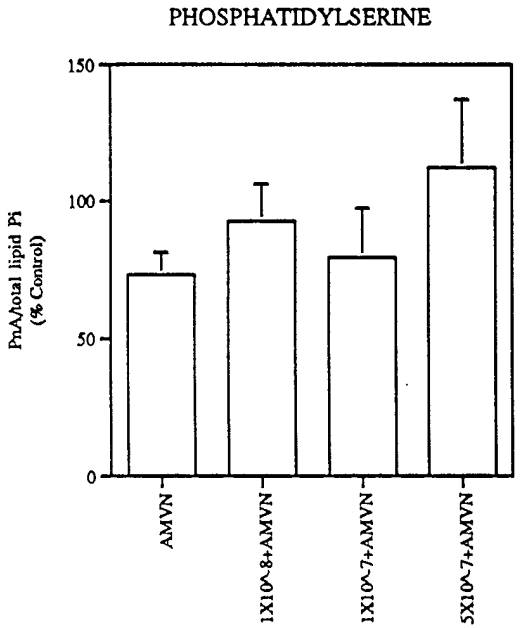
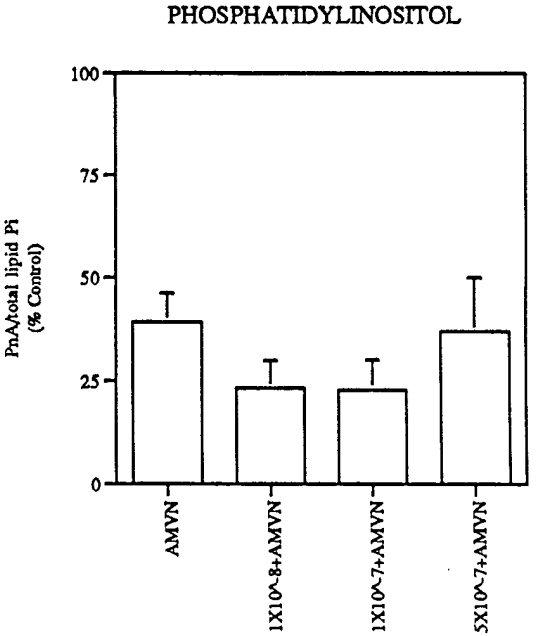
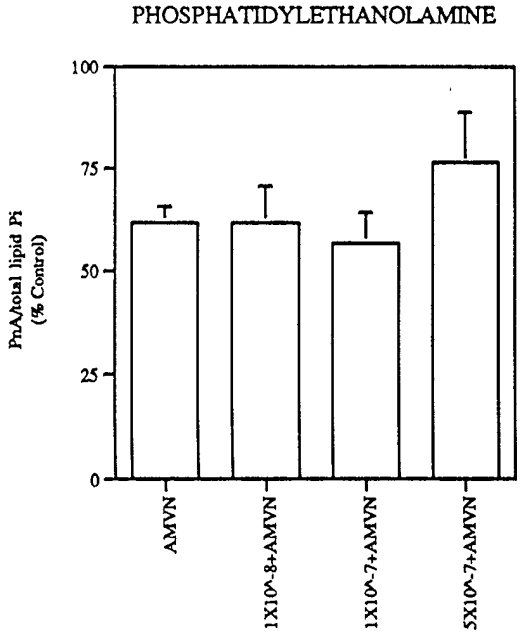

PHOSPHATIDYLCHOLINE
PnA/total lipid Pi
(% Control)
125
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN

Figure 2B


PHOSPHATIDYLSERINE
PnA/total lipid Pi
(% Control)
150
100
50
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN


PHOSPHATIDYLINOSITOL
PnA/total lipid Pi
(% Control)
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN


PHOSPHATIDYLETHANOLAMINE
PnA/total lipid Pi
(% Control)
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN

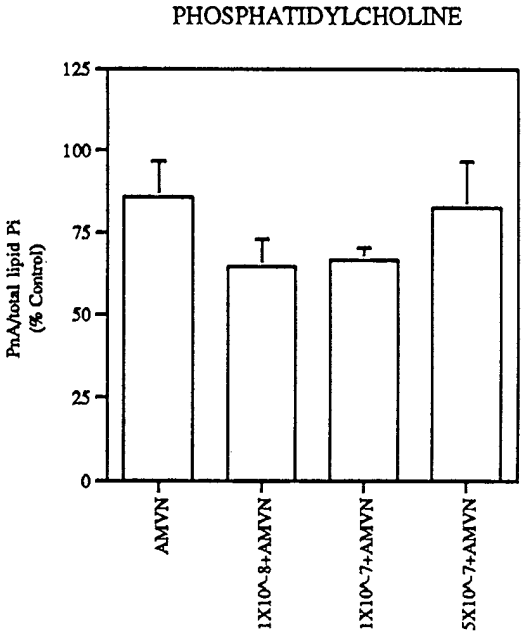
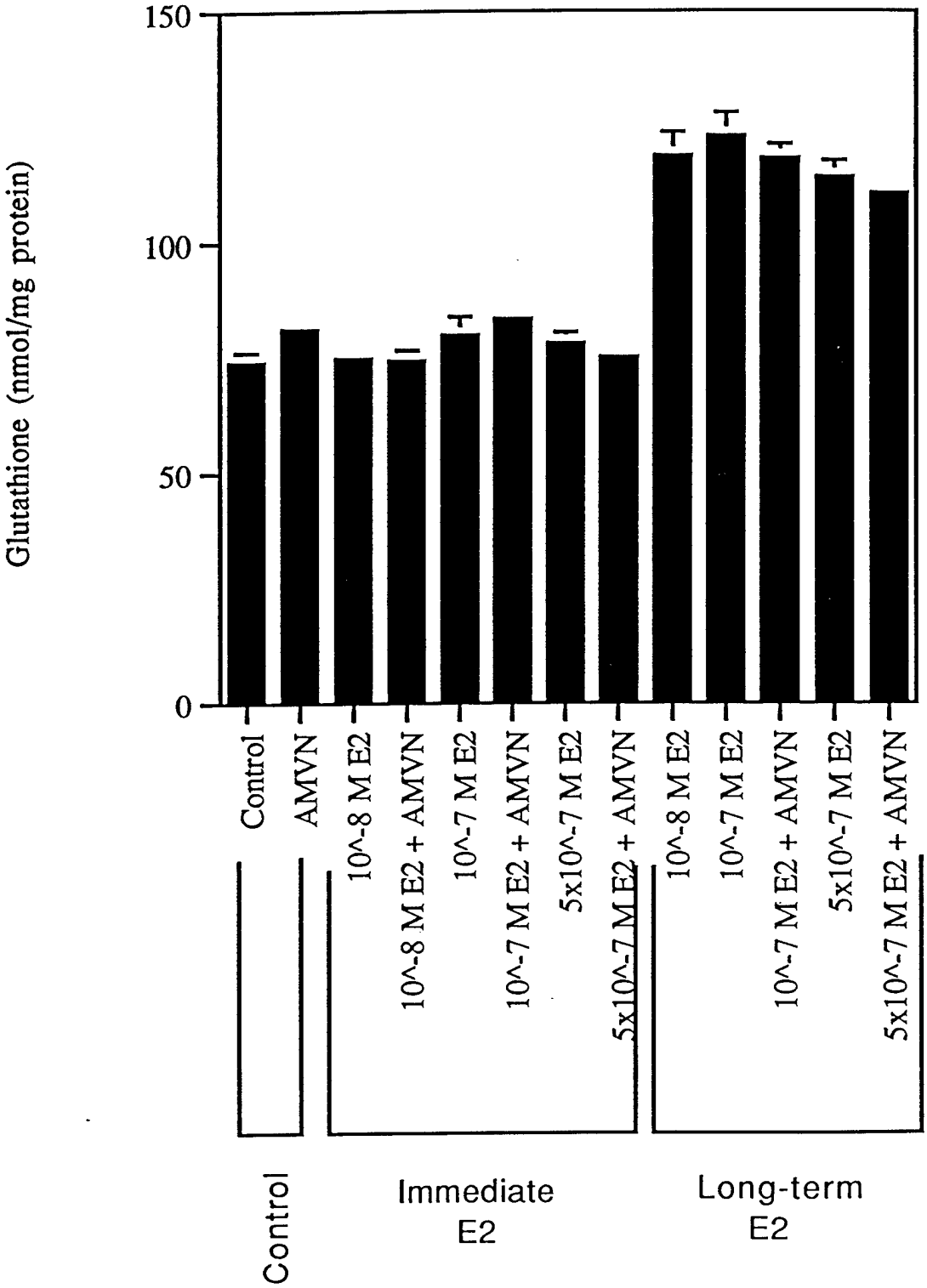

PHOSPHATIDYLCHOLINE
PnA/total lipid Pi
(% Control)
125
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN

Figure 3


Glutathione (nmol/mg protein)
150
100
50
0
Control
AMVN
10^-8 M E2
10^-8 M E2 + AMVN
10^-7 M E2
10^-7 M E2 + AMVN
5x10^-7 M E2
5x10^-7 M E2 + AMVN
10^-8 M E2
10^-7 M E2
10^-7 M E2 + AMVN
5x10^-7 M E2
5x10^-7 M E2 + AMVN
Control
Immediate E2
Long-term E2

Submitted to Molec. Pharmacol.

Bcl-2-INDUCED ALTERATION OF GLUTATHIONE HANDLING AND ITS EXPLOITATION IN TARGETED CANCER CHEMOTHERAPY

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RUNNING TITLE: GSH Handling and *bcl-2* Overexpression

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24 Text Pages

0 Tables

6 Figures

180 Words/Abstract

188 Words/Introduction

754 Words/Discussion

35 References

ABSTRACT

Bcl-2 has been associated with both oxidative and antioxidative effects in vivo. Moreover, despite evidence that Bcl-2 is antiapoptotic by virtue of its effect on reactive oxygen species and their scavengers, Bcl-2 exerts its antiapoptotic effects even under anaerobic conditions. The reasons for the variable relationship between Bcl-2 and reactive oxygen species are not clear. The present studies demonstrate that the impact of Bcl-2 on glutathione (GSH) metabolism is cell line-dependent. Bcl-2 overproduction in PC12 cells is associated with increased functional thiol reserves, increased reductive activation of chemotherapeutic prodrugs, and GSH accumulation after treatment with N-acetylcysteine. In contrast, Bcl-2-overproducing MCF-7 breast cancer cells demonstrate neither altered GSH handling nor potentiation of chemotherapeutic prodrug reduction. These findings indicate that the effects of Bcl-2 on GSH handling are millieu-dependent. This could account for the variable effects of Bcl-2 in in vivo systems. Furthermore, since our previous studies have demonstrated that reduction-dependent prodrugs may be useful chemotherapeutic agents against tumors that demonstrate altered GSH handling, screening in vitro for alteration of GSH handling may predict responsiveness of such tumors to these reduction-dependent agents.

INTRODUCTION

The induction and enactment of apoptosis have been associated with the generation of reactive oxygen species. In keeping with this association, overproduction of the antiapoptosis protein, Bcl-2, is associated in some systems with increased tolerance to oxidant stress (Kane et al., 1993; Albrecht et al., 1994; Tyurina et al., 1997), an increase in the cellular content of reducing species (Kane et al., 1993), and prevention of the release of cytochrome c from the mitochondrion (Yang et al., 1997; Kluck et al., 1997). However, in contradistinction to these studies, others have reported antiapoptosis activity of Bcl-2 in anaerobic systems (Jacobson and Raff, 1995) and have even ascribed a pro-oxidant function to Bcl-2 (Steinman, 1995).

Glutathione (GSH) is one of several antioxidant species the metabolism of which is thought to be altered by Bcl-2 overproduction. We examine herein the metabolism of GSH in native and bcl-2-transfected cell lines. Our results indicate that the implications of overproduction of Bcl-2 for GSH metabolism depend on the particular cell line involved. We illustrate this cell line-dependence and suggest its potential clinical application in our previously characterized (Cortazzo and Schor, 1996) chemotherapeutic model system.

MATERIALS AND METHODS

Chemicals and transfected cell lines: All chemicals used in enzyme assays were obtained from Sigma (St. Louis, MO). Media for tissue culture were obtained from GIBCO (Grand Island, NY). The enediyne antineoplastic agent, neocarzinostatin (NCS), was purchased as a powder from Kayaku Pharmaceuticals, Inc. (Tokyo, Japan). NCS is a reduction-dependent prodrug for a compound that induces apoptosis in cultured tumor cell lines (Cortazzo and Schor, 1996; Hartsell et al., 1995; Hartsell et al., 1996). Its intracellular activity is GSH-dependent (Cortazzo and Schor, 1996; DeGraff and Mitchell, 1985; DeGraff et al., 1985).

Mock- and bcl-2-transfected PC12 pheochromocytoma cells were prepared and isolated as a polyclonal population as previously published (Kane et al., 1993), and were the kind gift of Dr. Dale E. Bredesen (Burnham Institute, LaJolla, CA). Mock- and bcl-2-transfected MCF-7 human breast cancer cells were generated by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in Boise et al., 1993), respectively. Stably transfected MCF-7 clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology).

Enzyme assays: Mock- and bcl-2-transfected PC12 cells (10^7 of each) were harvested with trypsin from the tissue culture surface, washed free of medium, and suspended in 1 ml of phosphate-buffered saline. The suspension was homogenized and assayed for γ-glutamylcysteine synthetase (GCS) and glutathione reductase (GR) activities by modifications of the methods of Seelig and Meister (1985) and Carlberg and Mannervik (1985).

In brief, GCS activity was measured by coupling the generation of ADP by the enzyme to the oxidation of NADH by lactate dehydrogenase. Enzyme activity was determined at 37°C in a reaction mixture that contained 150 mM KCl, 5 mM ATP, 2 mM

phosphoenolpyruvate, 10 mM α-aminobutyrate, 10 mM L-glutamate, 20 mM $MgCl_2$, 2 mm Na_2EDTA, 0.2 mM NADH, 17 μg pyruvate kinase, and 17 μg lactate dehydrogenase in Tris-HCl buffer (0.1 M, pH 8.0). GR activity was measured by following the oxidation of NADPH by GSSG as the optical density of the assay mixture at 340 nm, as described by Carlberg and Mannervik (Carlburg and Mannervik, 1985). Each determination was performed in triplicate in each of two separate experiments. Values obtained for each experiment were compared between transfectants using Student's t test, with $p \leq 0.05$ considered statistically significant.

Glutathione efflux: The rate of efflux of glutathione from mock- and bcl-2-transfected cells was measured by determining the rate of decrease in intracellular glutathione concentration after complete inhibition of new glutathione synthesis. Cells were incubated with 1 mM BSO for the duration of the experiment, and total glutathione content of the cells was determined by the method of Tietze (1969) at 0.25, 0.5, 1, 4, 8, 12, and 24 hr after initiation of BSO treatment. Samples were analyzed in triplicate, and the mean ± SEM of each determination was plotted on a time course. The initial slope of the best fit curve for these points was taken as the efflux rate for glutathione from the cell. These slopes were compared between transfectants using Student's t test.

Effects of N-acetylcysteine on sensitivity of transfectants to NCS: N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 4 hr. Cells were washed free of extracellular N-acetylcysteine (to ensure that NCS did not get activated by this compound extracellularly), and an adherent cell number concentration-response curve to NCS was constructed for each transfectant as we have done in our previously published studies (Cortazzo and Schor, 1996). The ED_{50} for NCS was determined for each transfectant as we have previously done for apoptosis induction in

SH-SY5Y cells (Hartsell et al., 1995; Hartsell et al., 1996). The ED_{50}s obtained with and without N-acetylcysteine were compared for each transfectant using Student's t test, as we have previously described (Hartsell et al., 1996).

Effect of NCS on formation of glutathionyl radical: Mock- and bcl-2-transfected PC12 cells treated with varying concentrations of NCS were assayed for formation of the glutathionyl radical as a measure of glutathione turnover using the free radical trapping agent DMPO as we have previously described (Stoyanovsky et al., 1996). Briefly, cells were incubated with different amounts of NCS in the presence of DMPO (100 mM) for 1 hr at 37°C in the dark in medium (115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 10 mM glucose and 25 mM HEPES, pH 7.4). After incubation, cells were washed twice with medium, and GS-DMPO nitrone was determined by HPLC.

Effect of NCS on GSH and protein thiol content: Total protein sulfhydryl and GSH concentration in cells were determined using ThioGlo™ 1, a maleimide reagent that produces a highly fluorescent product upon its reaction with sulfhydryl groups (Langmuir et al., 1996). A standard curve was established by addition of GSH (0.04 - 4.0 μM) to 0.1 M phosphate buffer, pH 7.4, containing 200 μM ThioGlo™ 1 (from a stock solution in DMSO). GSH content was determined by an immediate fluorescence response registered upon addition of ThioGlo™ 1 to the cell homogenate. Total protein sulfhydryls were measured as an additional fluorescence response after addition of SDS (1.0 mM) to the same cell homogenate. A Shimadzu spectrofluorophotometer RF-5301PC was employed for these determinations using an excitation slit of 1.5 nm and an emission slit of 5 nm. The wavelengths employed in the assay were 388 nm (excitation) and 500 nm (emission). The data acquired were exported from the spectrofluorophotometer using RF-5301PC Personal Fluorescence Software (Shimadzu).

Effects of bcl-2 transfection on accumulation of glutathione in N-acetylcysteine-treated PC12 cells: N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 0.5 - 4 hr. Cells were washed free of extracellular N-acetylcysteine and assayed at various timepoints for total glutathione content by the spectrophotometric method of Tietze (1969).

RESULTS

Effects of bcl-2 transfection on glutathione accumulation in N-acetylcysteine-treated PC12 and MCF-7 cells: Incubation of mock-transfected PC12 cells with N-acetylcysteine (10 mM) for up to 8 hr results in a 3-fold increase in intracellular GSH that is apparent by 2 hr and plateaus until at least 4 hr of incubation (Fig. 1A). In contrast, similar incubation of bcl-2-transfected PC12 cells results in a GSH level 5-times baseline by 4 hr of treatment. Note that in these cultures that had not been fed for four days at t = 0, there was no significant difference in GSH content between mock- and bcl-2-transfected PC12 cells. Without additional N-acetylcysteine supplementation, GSH content of the bcl-2-transfected cells declines and the difference in GSH content between the two transfectants loses statistical signficance by 12 hr after addition of N-acetylcysteine to the medium (data not shown).

Analogous treatment of mock- and bcl-2-transfected MCF-7 cells with N-acetylcysteine resulted in GSH content kinetics that resembled those seen with mock-transfected PC12 cells (Fig. 1B). There was no significant difference between the MCF-7 bcl-2- and mock-transfectants in this regard.

Effect of bcl-2 transfection on GSH turnover in response to NCS treatment: Transfection of PC12 cells with bcl-2 results in GSH accumulation during incubation with N-acetylcysteine. Furthermore, the GSH content of bcl-2-transfected PC12 cells is augmented relative to native cells even in the absence of a supplemental cysteine source (Kane et al., 1993). This elevation of native GSH content and N-acetylcysteine-induced GSH accumulation could result from augmented GSH cycling in response to oxidative consumption. As such, we have examined the effects of bcl-2 transfection on the ability of PC12 cells to exchange and recycle GSH reducing equivalents (so-called "functional thiol reserves"; Hubel et al., 1997) in response to treatment with NCS, a known "consumer" of

GSH. We have done this by measuring the generation of glutathionyl radical (a mirror of oxidative GSH consumption) over a 1 hr incubation with NCS, and the cellular GSH content after such an incubation.

Treatment of bcl-2-transfected PC12 cells with NCS (0.2-0.6 nmol/10^6 cells) results in the trapping of 4 nmol/mg protein of glutathionyl radical over 1 hr (Fig. 2A). In a parallel study, analogous treatment of sister cultures results in a much smaller (< 2 nmol/mg protein) drop in the level of cellular GSH. In sharp contrast, treatment of mock-transfected PC12 cells with NCS results in no signficant change in the cumulative generation of glutathionyl radical or the cellular content of GSH (Fig. 2B).

Parallel studies of the effect of NCS treatment on cellular protein thiol content revealed no significant change in either PC12 transfectant (data not shown).

GSH-related enzyme activity and resting GSH efflux in mock- and bcl-2-transfected PC12 cells: In an effort to determine the mechanism of GSH accumulation and increased functional GSH reserves in bcl-2-transfected PC12 cells, we have measured the activities of GR and GCS in mock- and bcl-2-trasnfected PC12 cells both before and after 4 hr treatment with 10 mM N-acetylcysteine. As is shown in Fig. 3, there was no difference between the transfectants with respect to GCS activity either in the native state or after incubation with N-acetylcysteine. Furthermore, the observed lower GR activity in bcl-2-transfected PC12 cells than in mock-transfected cells is counter to the predicted increase in GR activity hypothesized to result in increased intracellular GSH levels after bcl-2 transfection.

The rate of efflux of GSH from native mock- and bcl-2-transfected PC12 cells was approximated by treatment of the cells with BSO (1 mM; to block new GSH synthesis) followed by timed measurement of total GSH per cell. There was no significant difference in the rate of decrease in the total cellular GSH content of the two transfectants after BSO treatment (Fig. 4).

Effects of bcl-2 transfection on the sensitivity of PC12 and MCF-7 cells to NCS: Our previous studies have demonstrated the paradoxical increase in sensitivity of PC12 rat pheochromocytoma cells to NCS afforded by bcl-2 transfection (Cortazzo and Schor, 1996; Fig. 5A). This potentiation of NCS toxicity was abrogated by prior incubation of PC12 cells with BSO. The difference in effect of bcl-2 transfection on GSH handling in PC12 and MCF-7 cells led us to predict that, in MCF-7 cells, the effects of bcl-2 transfection on apoptosis induced by reduction-dependent drugs like NCS would not differ from its effects on apoptosis induced by non-reduction-dependent drugs. That is, we predicted that bcl-2 transfection would prevent apoptosis in MCF-7 cells treated with NCS.

As predicted, unlike PC12 cells, bcl-2 overexpression protected MCF-7 cells from death induced by NCS (Fig. 5B). At each concentration, the survival of the bcl-2 transfectants exceeds that of the mock transfectants. Note also that mock-transfected MCF-7 cells are 10-fold more sensitive than mock-transfected PC12 cells to the effects of NCS.

Effect of bcl-2-transfection on potentiation of NCS-induced apoptosis by incubation with N-acetylcysteine: In light of the accumulation of GSH seen only in bcl-2-transfected PC12 cells after incubation with N-acetylcysteine, and the concentration-dependent role of GSH in activation of NCS (DeGraff and Mitchell, 1985; DeGraff et al., 1985; Beerman et al., 1977; Schor, 1992), we hypothesized that pretreatment of bcl-2-transfected PC12 cells with N-acetylcysteine would result in further potentiation of NCS-induced apoptosis, while the response of mock-transfected PC12 cells to NCS would remain unaltered. Fig. 6A demonstrates the shift in the concentration-response curve of bcl-2-transfected PC12 cells to NCS after a 4 hr incubation with N-acetylcysteine. In contrast, the concentration-response curve of bcl-2-transfected MCF-7 cells to NCS is unaffected by N-acetylcysteine treatment (Fig. 6B). The sensitivity to NCS of mock-transfected cells of both types is unaffected by N-acetylcysteine exposure (data not shown).

DISCUSSION

The induction of apoptosis in neural cells has been hypothesized to play a role in the morphological and physiological changes associated with a variety of developmental and pathological states (Narayanan, 1997; Gelbard et al., 1997). For many of these, reactive oxygen species (ROS) have been implicated in the disease pathogenesis itself and/or as final common mediators of apoptosis (Wood and Youle, 1994; Wood and Youle, 1995). Similarly, species known to influence the induction and enactment of apoptosis have been proposed as modulators of development and disease and cellular handling of ROS.

Some studies of the effects of bcl-2 overexpression on ROS handling in neural cells have suggsted a role for Bcl-2 in increasing the tolerance of such cells to oxidant stress (Kane et al., 1993; Albrecht et al., 1994; Tyurina et al., 1997). Conversely, some studies have proposed a pro-oxidant role for Bcl-2, and view the enrichment of the intracellular reducing potential as a compensatory, cell-generated phenomenon (Steinman, 1995). Still others have pointed out the Bcl-2 overproduction is protective from apoptosis under near-anaerobic conditions (Jacobson and Raff, 1995), and that GSH depletion does not abolish the protective effects of Bcl-2 (Kane et al., 1993). The present results demonstrate the cell-dependence of the effects of Bcl-2 on GSH metabolism. The impact of Bcl-2 on the reducing potential of the cell is millieu-dependent. This may contribute to the variability of the involvement of ROS and their scavenging in the enactment and prevention of apoptosis.

The determination by the intracellular environment of the redox activity of Bcl-2 and/or the species that are produced or altered as a result of its expression is not unique. The potential for other redox-active agents, including vitamins C and E, to either potentiate or inhibit oxidation is well documented (Halliwell and Gutteridge, 1985).

Our studies of NCS treatment of PC12 pheochromocytoma and MCF-7 breast cancer cells demonstrate the consequences of this biochemical variability and exemplify the potential therapeutic importance of our findings. We have previously reported that, unlike

the case for other chemotherapeutic agents from which cells are protected by Bcl-2 (Dole et al., 1994; Dole et al., 1995; Teixeira et al., 1995; Bonetti et al., 1996; Beham et al., 1996; Campos et al., 1993; Reber et al, 1998), Bcl-2 potentiates the induction of apoptosis by the enediyne NCS (Cortazzo and Schor, 1996). From the mechanistic standpoint, the present results suggest increased intracellular activation of NCS in bcl-2-overexpressing cells, as evidenced by increased production of the glutathionyl radical. The relatively small concentration ratio of added NCS in the bathing medium to GSH intracellularly may be deceptive, since NCS is actively taken up into cells by endocytosis and may therefore be considerably more concentrated intracellularly than extracellularly. Additional increments in NCS concentration may arise from specific compartmentalization of this compound within cellular organelles (Maeda, 1984). In addition, our finding that the rate of turnover of GSH is higher in Bcl-2-overproducing PC12 cells than in native producers exposed to NCS implies that not only the endogenous rate of production of ROS, but also the role of GSH in and ability to compensate for consumption of reducing equivalents is aberrant in some bcl-2-overexpressing cells. This increased GSH turnover, as evidenced by increased formation of the glutathionyl radical, is not related to changes in GSH peroxidase activity, as such changes are not associated with glutathionyl radical formation (Stoyanovsky et al., 1996). They are rather related to the non-enzymatic generation of glutathionyl radical via interaction with NCS (Stoyanovsky et al., 1996; Chin et al., 1988). This finding, along with our previous observation that the downstream block in the apoptosis final common pathway produced by Bcl-2 is lifted in NCS-treated cells (Schor et al., 1999), explains the enhanced apoptotic rate in bcl-2-transfected PC12 cells treated with the reduction-dependent prodrug, NCS. The exploitation by NCS of the effects of Bcl-2 on GSH handling in some cells make NCS a potential chemotherapeutic drug for these chemoresistant tumors. However, the cell-dependent effects of Bcl-2 on GSH handling imply that Bcl-2 content alone could not be used as a criterion for predicting the efficacy of NCS against tumor cells.

That incubation with N-acetylcysteine accentuates the difference in GSH handling between bcl-2- and mock-transfected PC12 cells, but not MCF-7 cells, suggests that such incubation would augment the potentiation of apoptosis in bcl-2-overexpressing PC12 cells, but not in the analogous MCF-7 cells. Indeed, our results bear this out, suggesting that an in vitro assay of GSH accumulation after N-acetylcysteine incubation might be developed to predict the likely responsiveness of a particular tumor to NCS.

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REFERENCES

Albrecht, H., Tschopp, J., and Jongeneel, C.V. (1994) Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-κB by TNF. *FEBS Lett* **351**:45-48.

Beerman, T. A., Poon, R., and Goldberg, I. H. (1977) Single-strand nicking of DNA in vitro by neocarzinostatin and its possible relationship to the mechanism o drug action. *Biochim. Biophys. Acta* **475**:294-306.

Beham, A. W., and McDonnell, T. J. (1996) Bcl-2 confers resistance to androgen deprivation in prostate carcinoma cells. Proc Amer Assoc Cancer Research **37**:224.

Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**:597-608.

Bonetti, A., Zaninelli, M., Pavanel, F., Sperotto, L., Molino, A., Pelosi, G., Cetto, G. L., Biolo, S., and Piubello, Q. (1996) Bcl-2 expression is associated with resistance to chemotherapy in advanced breast cancer. *Proc Amer Assoc Cancer Research* **37**:192.

Campos, L., Rouault, J.-P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J.-P., and Guyotat, D. (1993) High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* **81**:3091-3096.

Carlburg, I., and Mannervik, B. (1985) Glutathione reductase. *Meth Enzymol* **113**:484-489.

Chin, D.-H., Zeng, C.-H., Costello, C. E., and Goldberg, I. H. (1988) Sitesin the diyne-ene bicyclic core of neocarzinostatin chromophore responsible for hydrogen abstraction from DNA. *Biochemistry* **27**:8106-8114.

Cortazzo, M., and Schor, N. F. (1996) Potentiation of enediyne-induced apoptosis and differentiation by Bcl-2. *Cancer Research* **56**:1199-1203.

DeGraff, W. G. and Mitchell, J. B. (1985) Glutathione dependence of neocarzinostatin cytotoxicity and mutagenicity in Chinese hamster V-79 cells. *Cancer Research* **45**:4760-4762.

DeGraff, W. G., Russo, A., and Mitchell, J. B. (1985) Glutathione depletion greatly reduces neocarzinostatin cytotoxicity in Chinese hamster V-79 cells. *J Biol Chem* **260**:8312-8315.

Dole, M., Nunez, G., Merchant, A. K., Maybaum, J., Rode, C. K., Bloch, C. A., and Castle, V. P. (1994) Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. *Cancer Research* **54**:3253-3259.

Dole, M. G., Jasty, R., Cooper, M. J., Thompson, C. B., Nunez, G., and Castle, V. P. (1995) Bcl-x_L is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. *Cancer Research* **55**:2576-2582.

Gelbard, H. A., Boustany, R. M., and Schor, N. F. (1997) Apoptosis in development and disease of the nervous system: II. apoptosis in childhood neurologic death. *Pediatr Neurol* **16**:93-97.

Halliwell, B., and Gutteridge, J. M. C. (1985) Oxygen radicals and the nervous system. *TINS* **8**:22-26.

Hartsell, T. L., Yalowich, J. C., Ritke, M., Martinez, A. J., and Schor, N. F. (1995) Induction of apoptosis in murine and human neuroblastoma cell lines by the enediyne natural product neocarzinostatin. *J Pharm Exp Therap* **275**:479-485.

Hartsell, T. L., Hinman, L. M., Hamann, P. R., and Schor, N. F. (1996) Determinants of the response of neuroblastoma cells to DNA damage: the roles of pre-treatment cell morphology and chemical nature of the damage. *J Pharm Exp Therap* **277**:1158-1166.

Hubel, C. A., Kagan, V. E., Kisin, E. R., McLaughlin, M. K., and Roberts, J. M. (1997) Increased ascorbate radical formation and ascorbate depletion in plasma from women with pre-eclampsia: implications for oxidative stress. *Free Radical Biol Med.***23**:596-609.

Jacobson, M. D. and Raff, M. C. (1995) Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* **374**:814-816.

Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T., and Bredesen, D. E. (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* **262**:1274-1277.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D.D. (1997) The release of cytochrome c from mitochonria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**:1132-1136.

Langmuir, M. E., Yang, J.-R., LeCompte, K. A., and Durand, R. E. (1996) In: *Fluorescence Microscopy and Fluorescent Probes* Plenum Press, NY, NY, pp. 229-234.

Maeda, H. (1994) The clinical effects of neocarzinostatin and its polymer conjugate, SMANCS. In: *Enediyne Antibiotics as Antitumor Agents* Marcel Decker, NY, NY, pp.363-381.

Narayanan, V. (1997) Apoptosis in development and disease of the nervous system: I. naturally occurring cell death in the developing nervous system. *Pediatr Neurol.***16**:9-13.

Reber, U., Wullner, U., Trepel, M., Baumgart, J., Seyfried, J., Klockgether, T., Dichgans, J., and Weller, M. (1998) Potentiation of treosufan toxicity by the glutathione-depleting agent buthionine sulfoximine in human malignant glioma cells: the role of Blc-2. *Biochem Pharmacol* **55**:349-359.

Schor, N. F. (1992) Targeted enhancement of the biological activity of the antineoplastic agent, neocarzinostatin. *J Clin Invest* **89**:774-781.

Seelig, G. F., and Meister, A. (1985) Glutathione biosynthesis; γ-glutamylcysteine synthetase from rat kidney. *Meth Enzymol* **113**:379-389.

Steinman, H. M. (1995) The Bcl-2 oncoprotein functions as a pro-oxidant. *J Biol Chem* **270**:3487-3490.

Stoyanovsky, D. A., Goldman, R., Jonnalagadda, S. S., Day, B. W., Claycamp, H. G., and Kagan, V. E. (1996) Detection and characterization of the electron paramagnetic resonance-silent glutathionyl-5,5-dimethyl-1-pyrroline N-oxide adduct derived from redox cycling of phenoxyl radicals in model systems and HL-60 cells. *Arch Biochem Biophys*.**330**:3-11.

Teixeira, C., Reed, J. C., and Pratt, M. A. (1995) Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 protooncogene expression in human breast cancer cells. *Cancer Research* **55**:3902-3907.

Tietze, F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Analyt Biochem* **27**:502-522.

Tyurina, Y. Y., Tyurin, V. A., Carta, G., Quinn, P. J., Schor, N. F., and Kagan, V. E. (1997) Direct evidence for antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cels. *Arch Biochem Biophys* **344**:412-433.

Wood, K. A., and Youle, R. J. (1994) Apoptosis and free radicals. *Ann NY Acad Sci* **738**:400-407.

Wood, K. A., and Youle, R. J. (1995) The role of free radicals and p53 in neuron apoptosis in vivo. *J Neurosci* **15**:5851-5857.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**:1129-1132.

FOOTNOTES

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FIGURE LEGENDS

Fig. 1: Effects of bcl-2 transfection on accumulation of GSH in tumor cells continuously incubated with N-acetylcysteine (NAcCys; 10 mM). GSH was measured for the results depicted using the method of Tietze (1969). Independently-obtained samples assayed using the Thio-Glo™ method (Langmuir et al., 1996) gave analogous results. (A) PC12 pheochromocytoma cells. Four independent experiments were performed and gave comparable results. The results of duplicate determinations from one of these experiments are shown. (B) MCF-7 breast cancer cells. The results of single determinations from each of two mock- (Neo.1-mock and Neo.2-mock) and two bcl-2-transfected (Bcl-2.1 and Bcl-2.2) clones of MCF-7 cells are shown. A second independent experiment gave comparable results.

Fig. 2: Effect of NCS on the formation of GS-DMPO nitrone in mock- (B) and bcl-2- (A) transfected PC12 pheochromocytoma cells. Cells were incubated with different amounts of NCS in the presence of DMPO (100 mM) for 1 hr at 37 °C in the dark in medium. After incubation, cells were washed twice with medium, and GS-DMPO nitrone and GSH content were determined as described in Experimental Procedures. Results shown are means ± SEM of three independent experiments.

Fig. 3: Activity of GR and GCS in native and N-acetylcysteine-treated (10 mM; 4 hr; 37°C) mock- and bcl-2-transfected PC12 pheochromocytoma cells. Results shown are means ± SEM from triplicate determinations of the change in OD measured in each of the assays (Seelig and Meister, 1985; Carlburg and Mannervik, 1985) on homogenates from 10^7 cells.

Fig. 4: Effect of bcl-2 transfection on the native efflux rate of GSH from PC12 pheochromocytoma cells. Mock- and bcl-2-transfected PC12 cells were continuously treated with BSO (1 mM) and GSH was measured at various times after BSO addition. Data shown represent the means ± SEM from triplicate determinations.

Fig. 5: Effects of bcl-2 transfection on sensitivity of cultured tumor cells to NCS. In all cases, cells were treated for 1 hr with NCS (37 °C) on day 0 and counted daily as described previously (Cortazzo and Schor, 1996). Points represent the mean of counts from three separate high power fields expressed as a percent of the count on day 0. Error bars signify the SEM. (A) PC12 pheochromocytoma cells; (B) MCF-7 breast cancer cells. Transfection and selection of these lines is described in Experimental Procedures.

Fig. 6: Effect of N-acetylcysteine (10 mM; 37°C; 4 hr) pretreatment on NCS sensitivity of PC12 pheochromocytoma cells (A) and MCF-7 cells (B). NCS treatment (1 hr; 37°C) was performed on sister cultures immediately after washout of N-acetylcysteine (NAcCys) or vehicle (no NAcCys) from the medium. Results shown represent the mean cell counts of three determinations from one of two independent and comparable experiments. SEMs of the triplicate determinations are plotted but in some cases are too small to be resolved on the plot. Adherent cell counts are shown during mid-log-phase growth (day 5 for PC12 cells and day 3 for MCF-7 cells).

Figure 1A

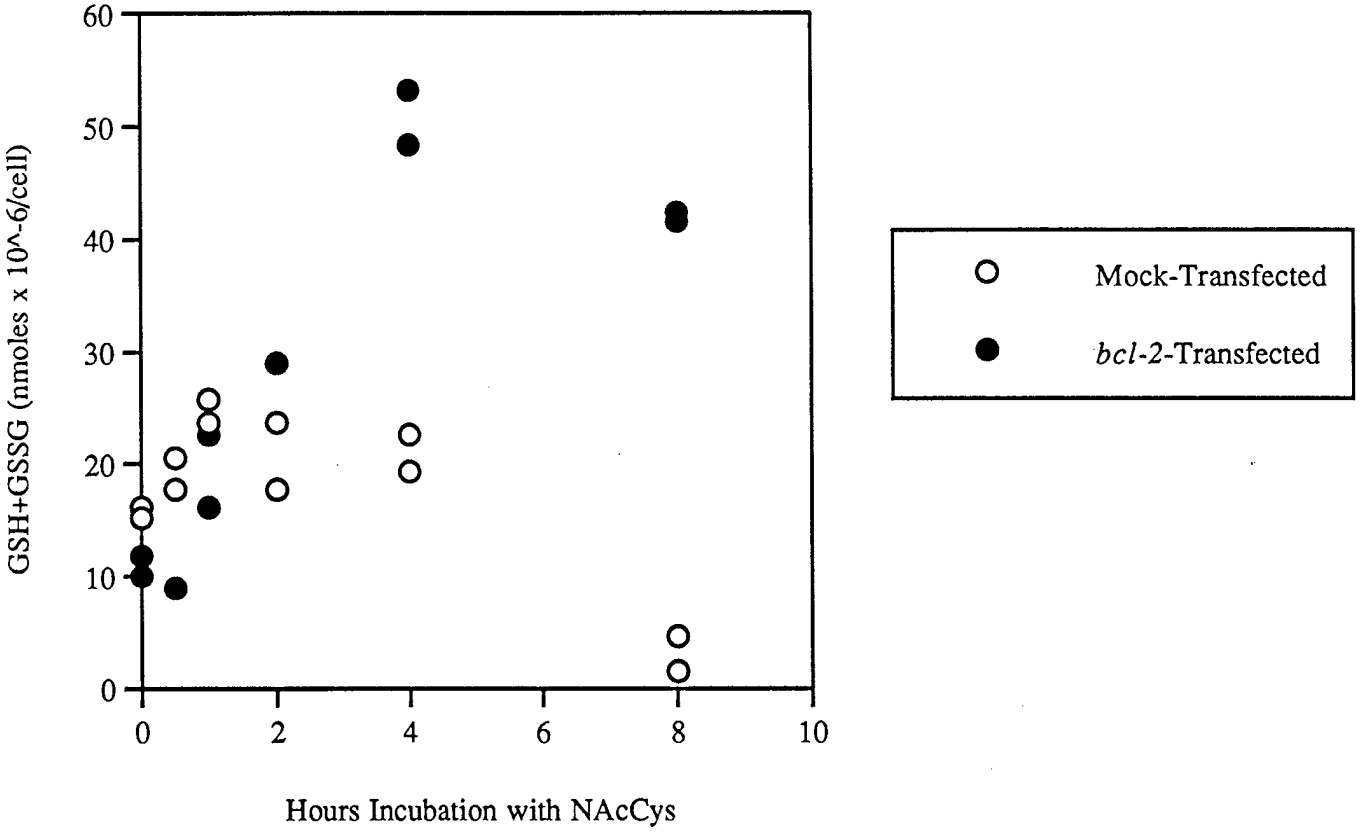

60
50
40
30
20
10
0
0
2
4
6
8
10
GSH+GSSG (nmoles x 10^-6/cell)
Hours Incubation with NAcCys
Mock-Transfected
bcl-2-Transfected

Figure 1B

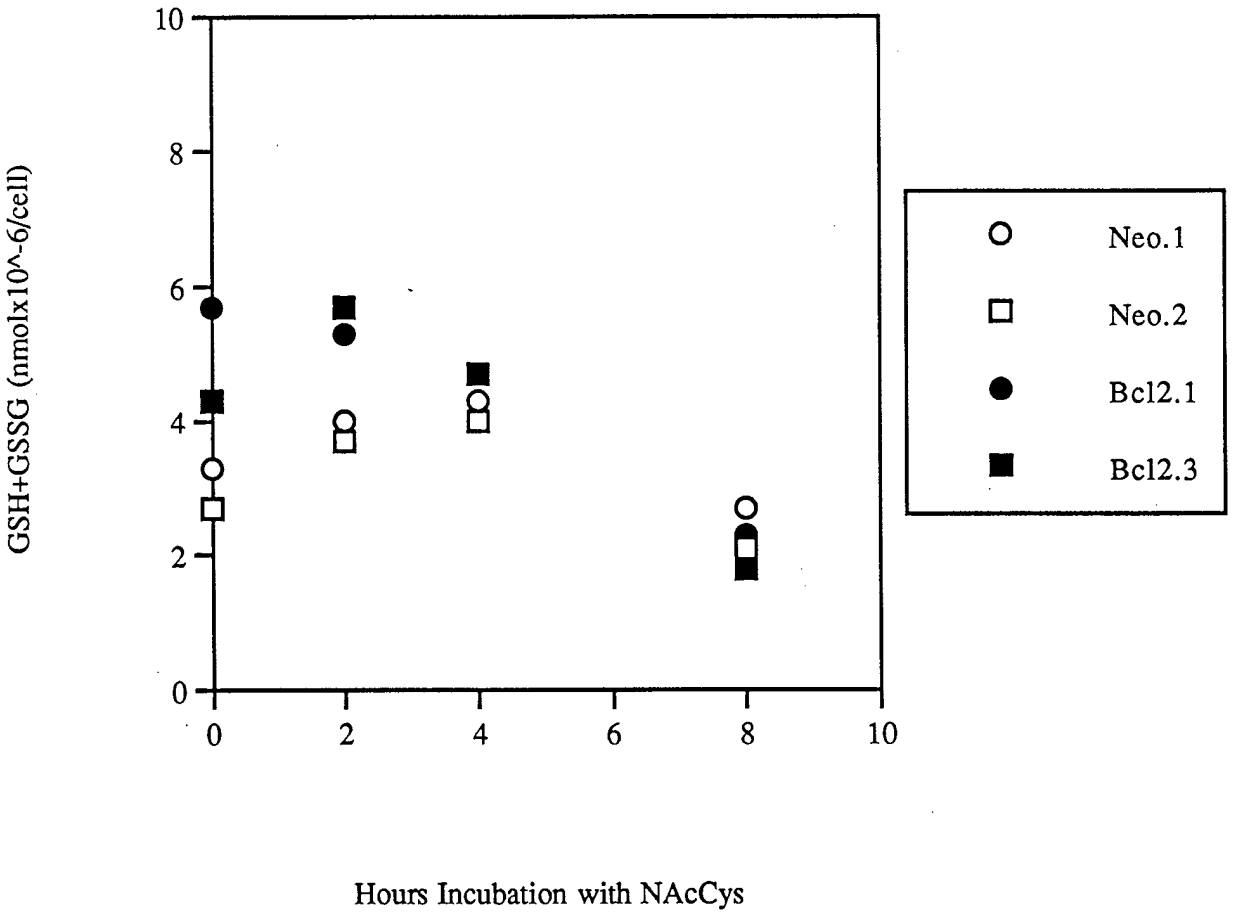

GSH+GSSG (nmolx10^-6/cell)
10
8
6
4
2
0
0
2
4
6
8
10
Hours Incubation with NAcCys
Neo.1
Neo.2
Bcl2.1
Bcl2.3

Figure 2A

bcl-2-TRANSFECTED PC12 CELLS

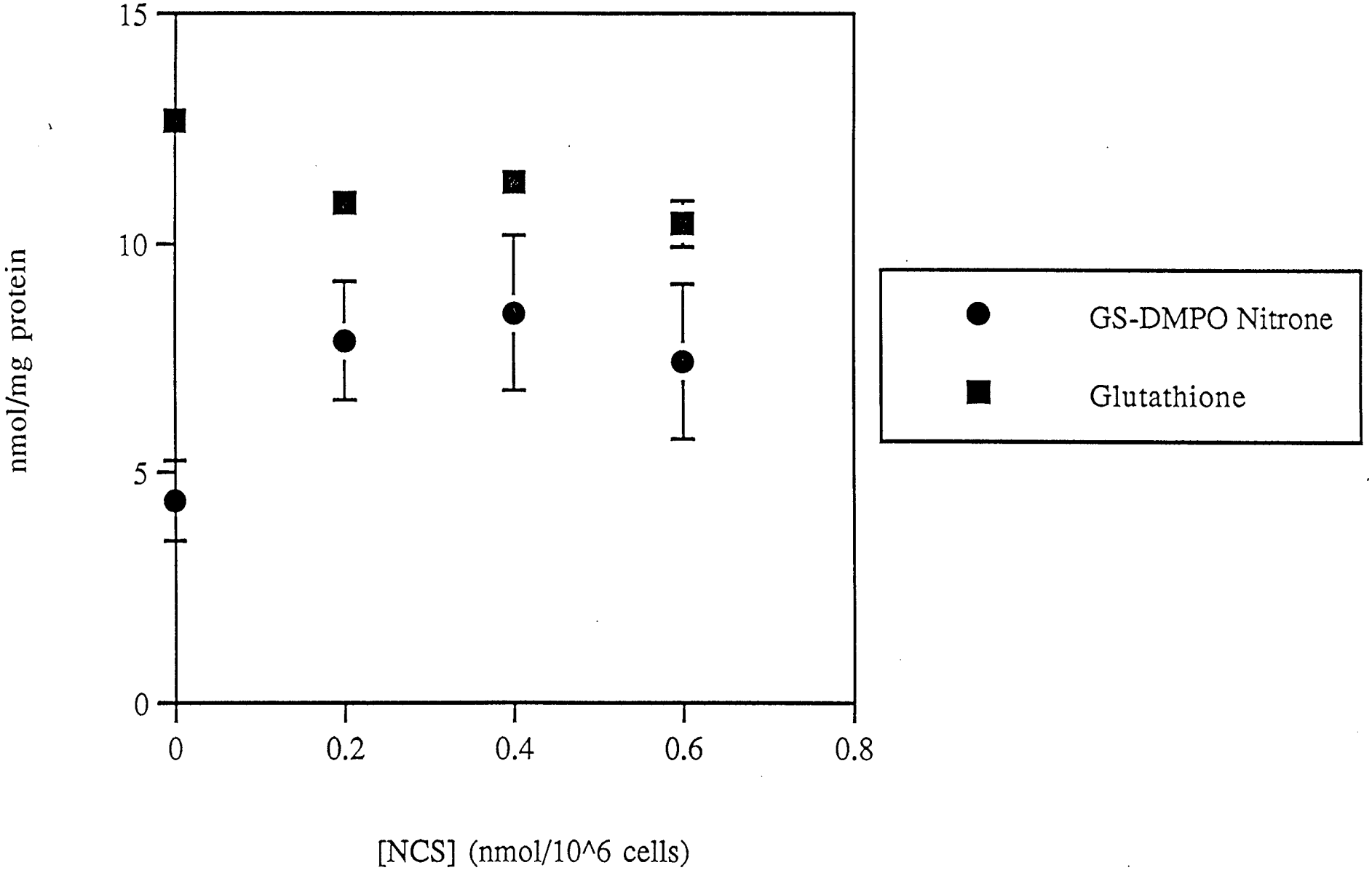


Figure 2B

MOCK-TRANSFECTED PC12 CELLS

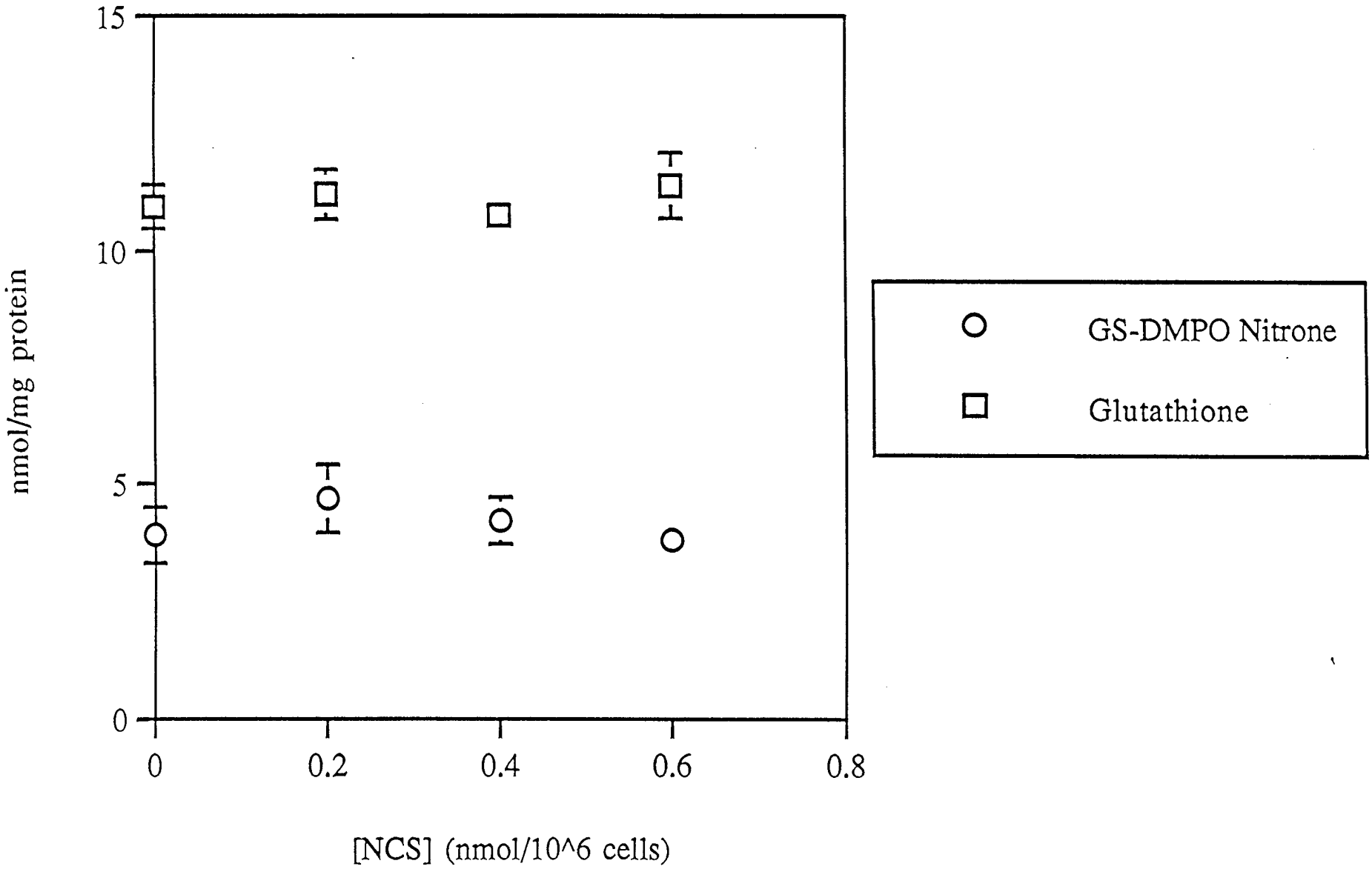


Figure 3

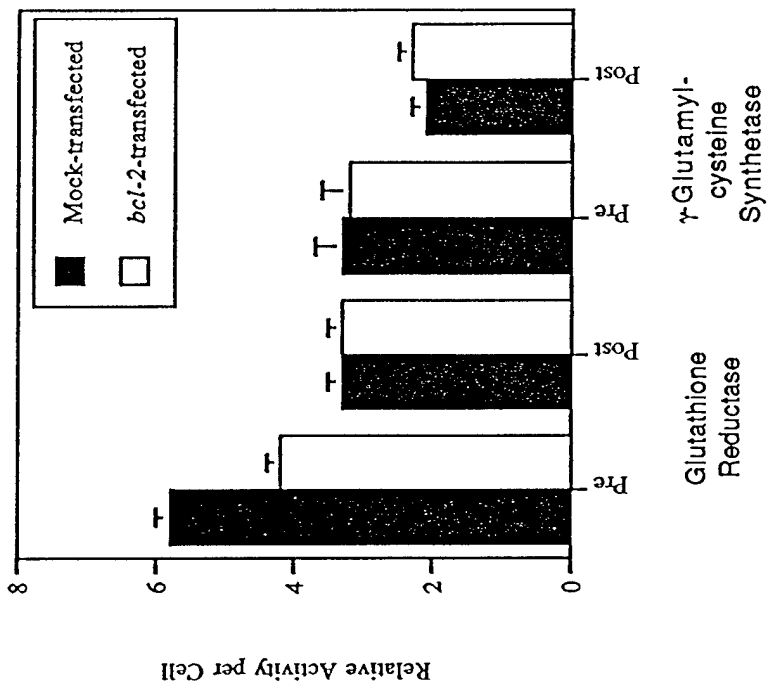

Mock-transfected
bcl-2-transfected
Relative Activity per Cell
0
2
4
6
8
Pre
Post
Pre
Post
Glutathione Reductase
γ-Glutamyl-cysteine Synthetase

Figure 4

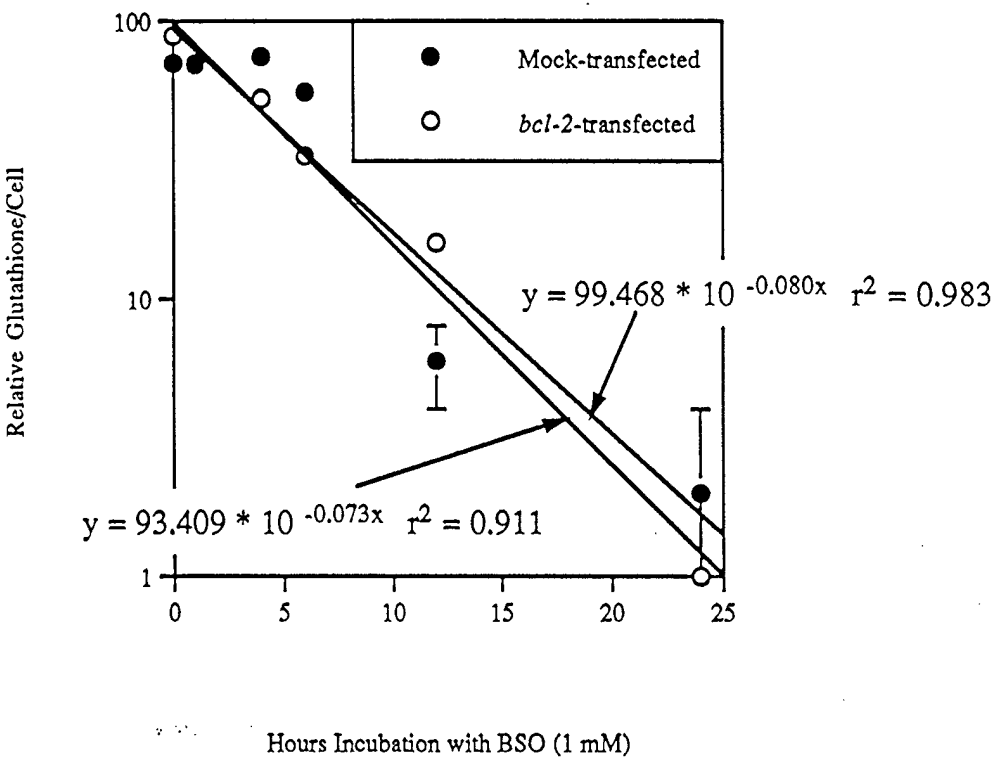

100
10
1
0
5
10
15
20
25
Mock-transfected
bcl-2-transfected
y = 99.468 * 10 -0.080x r2 = 0.983
y = 93.409 * 10 -0.073x r2 = 0.911
Relative Glutathione/Cell
Hours Incubation with BSO (1 mM)

Figure 5A

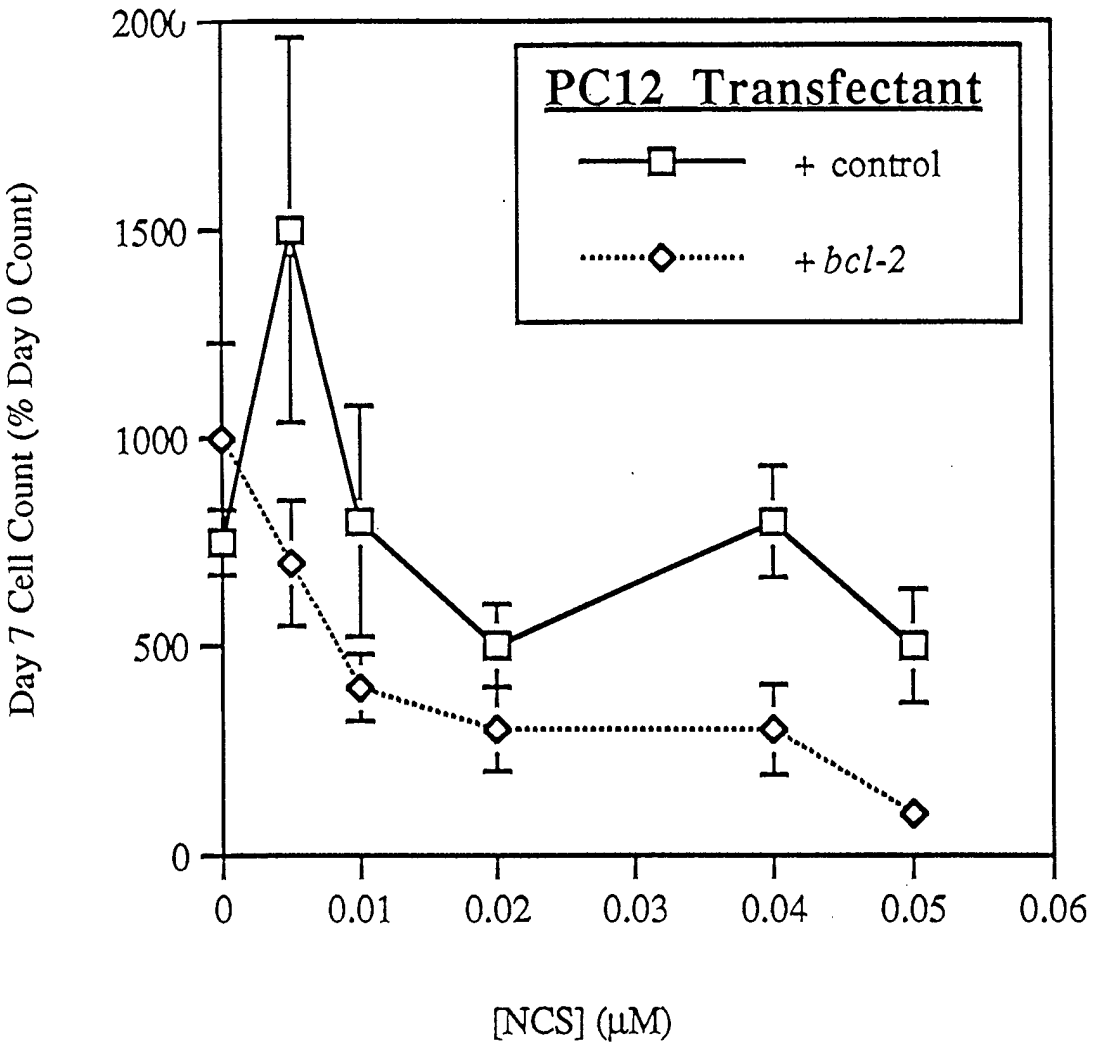

PC12 Transfectant
+ control
+ bcl-2
Day 7 Cell Count (% Day 0 Count)
2000
1500
1000
500
0
0
0.01
0.02
0.03
0.04
0.05
0.06
[NCS] (μM)

Figure 5B

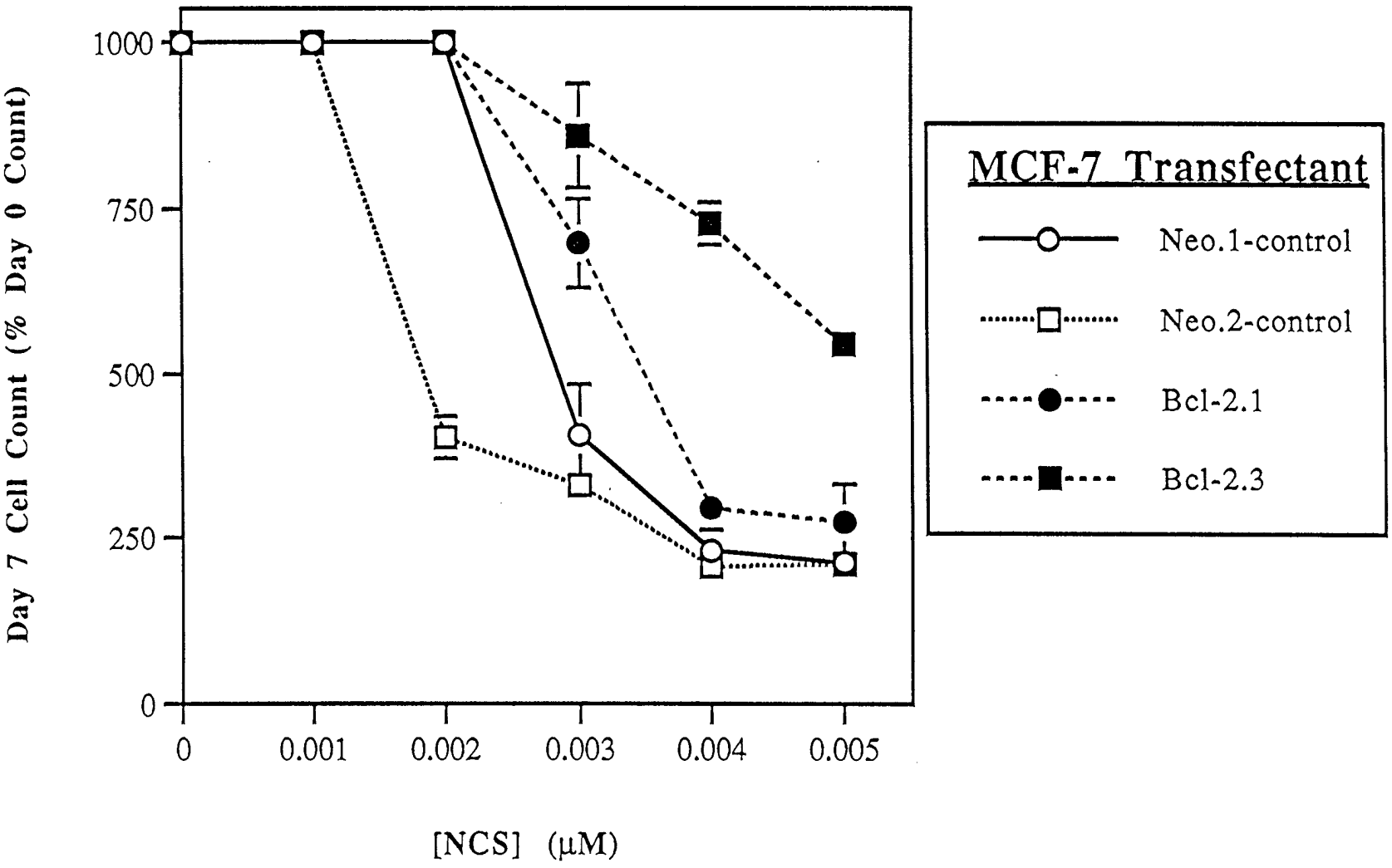

Day 7 Cell Count (% Day 0 Count)
1000
750
500
250
0
0
0.001
0.002
0.003
0.004
0.005
[NCS] (μM)
MCF-7 Transfectant
Neo.1-control
Neo.2-control
Bcl-2.1
Bcl-2.3

Figure 6A

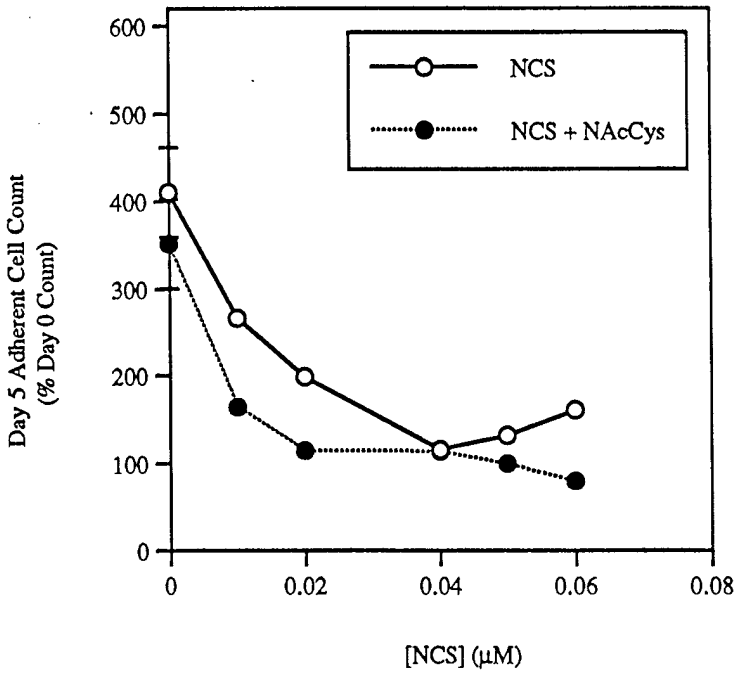
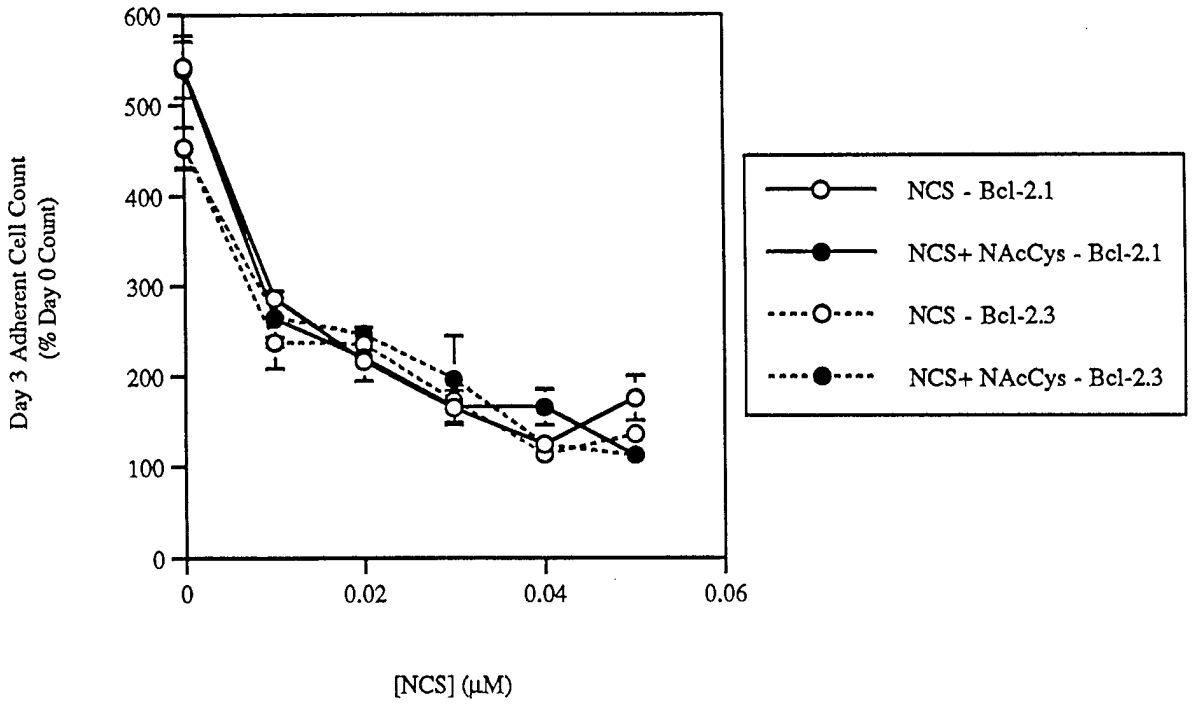

600
500
400
300
200
100
0
0
0.02
0.04
0.06
0.08
NCS
NCS + NAcCys
Day 5 Adherent Cell Count
(% Day 0 Count)
[NCS] (μM)

Figure 6B


600
500
400
300
200
100
0
0
0.02
0.04
0.06
Day 3 Adherent Cell Count
(% Day 0 Count)
[NCS] (µM)
NCS - Bcl-2.1
NCS+ NAcCys - Bcl-2.1
NCS - Bcl-2.3
NCS+ NAcCys - Bcl-2.3

REFERENCES

1. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T., and Bredesen, D. E. (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* **262**:1274-1277.

2. Albrecht, H., Tschopp, J., and Jongeneel, C.V. (1994) Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-κB by TNF. *FEBS Lett* **351**:45-48.

3. Tyurina, Y. Y., Tyurin, V. A., Carta, G., Quinn, P. J., Schor, N. F., and Kagan, V. E. (1997) Direct evidence for antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cels. *Arch Biochem Biophys* **344**:412-433.

4. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**:1129-1132.

5. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D.D. (1997) The release of cytochrome c from mitochonria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**:1132-1136.

6. Jacobson, M. D. and Raff, M. C. (1995) Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* **374**:814-816.

7. Steinman, H. M. (1995) The Bcl-2 oncoprotein functions as a pro-oxidant. *J Biol Chem* **270**:3487-3490.

8. Cortazzo, M., and Schor, N. F. (1996) Potentiation of enediyne-induced apoptosis and differentiation by Bcl-2. *Cancer Research* **56**:1199-1203.

9. Hartsell, T. L., Yalowich, J. C., Ritke, M., Martinez, A. J., and Schor, N. F. (1995) Induction of apoptosis in murine and human neuroblastoma cell lines by the enediyne natural product neocarzinostatin. *J Pharm Exp Therap* **275**:479-485.

10. Hartsell, T. L., Hinman, L. M., Hamann, P. R., and Schor, N. F. (1996) Determinants of the response of neuroblastoma cells to DNA damage: the roles of pre-treatment cell morphology and chemical nature of the damage. *J Pharm Exp Therap* **277**:1158-1166.

11. DeGraff, W. G. and Mitchell, J. B. (1985) Glutathione dependence of neocarzinostatin cytotoxicity and mutagenicity in Chinese hamster V-79 cells. *Cancer Research* **45**:4760-4762.

12. DeGraff, W. G., Russo, A., and Mitchell, J. B. (1985) Glutathione depletion greatly reduces neocarzinostatin cytotoxicity in Chinese hamster V-79 cells. *J Biol Chem* **260**:8312-8315.

13. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**:597-608.

14. Seelig, G. F., and Meister, A. (1985) Glutathione biosynthesis; γ-glutamylcysteine synthetase from rat kidney. *Meth Enzymol* **113**:379-389.

15. Carlburg, I., and Mannervik, B. (1985) Glutathione reductase. *Meth Enzymol* **113**:484-489.

16. Tietze, F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Analyt Biochem* **27**:502-522.

17. Stoyanovsky, D. A., Goldman, R., Jonnalagadda, S. S., Day, B. W., Claycamp, H. G., and Kagan, V. E. (1996) Detection and characterization of the electron paramagnetic resonance-silent glutathionyl-5,5-dimethyl-1-pyrroline N-oxide adduct derived from redox cycling of phenoxyl radicals in model systems and HL-60 cells. *Arch Biochem Biophys*.**330**:3-11.

18. Langmuir, M. E., Yang, J.-R., LeCompte, K. A., and Durand, R. E. (1996) In: *Fluorescence Microscopy and Fluorescent Probes* Plenum Press, NY, NY, pp. 229-234.

19. Hubel, C. A., Kagan, V. E., Kisin, E. R., McLaughlin, M. K., and Roberts, J. M. (1997) Increased ascorbate radical formation and ascorbate depletion in plasma from women with pre-eclampsia: implications for oxidative stress. *Free Radical Biol Med*.**23**:596-609.

20. Beerman, T. A., Poon, R., and Goldberg, I. H. (1977) Single-strand nicking of DNA in vitro by neocarzinostatin and its possible relationship to the mechanism o drug action. *Biochim. Biophys. Acta* **475**:294-306.

21. Schor, N. F. (1992) Targeted enhancement of the biological activity of the antineoplastic agent, neocarzinostatin. *J Clin Invest* **89**:774-781.

22. Narayanan, V. (1997) Apoptosis in development and disease of the nervous system: I. naturally occurring cell death in the developing nervous system. *Pediatr Neurol.***16**:9-13.

23. Gelbard, H. A., Boustany, R. M., and Schor, N. F. (1997) Apoptosis in development and disease of the nervous system: II. apoptosis in childhood neurologic death. *Pediatr Neurol* **16**:93-97.

24. Wood, K. A., and Youle, R. J. (1994) Apoptosis and free radicals. *Ann NY Acad Sci* **738**:400-407.

25. Wood, K. A., and Youle, R. J. (1995) The role of free radicals and p53 in neuron apoptosis in vivo. *J Neurosci* **15**:5851-5857.

26. Dole, M., Nunez, G., Merchant, A. K., Maybaum, J., Rode, C. K., Bloch, C. A., and Castle, V. P. (1994) Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. *Cancer Research* **54**:3253-3259.

27. Dole, M. G., Jasty, R., Cooper, M. J., Thompson, C. B., Nunez, G., and Castle, V. P. (1995) Bcl-x_L is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. *Cancer Research* **55**:2576-2582.

28. Teixeira, C., Reed, J. C., and Pratt, M. A. (1995) Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 protooncogene expression in human breast cancer cells. *Cancer Research* **55**:3902-3907.

29. Bonetti, A., Zaninelli, M., Pavanel, F., Sperotto, L., Molino, A., Pelosi, G., Cetto, G. L., Biolo, S., and Piubello, Q. (1996) Bcl-2 expression is associated with resistance to chemotherapy in advanced breast cancer. *Proc Amer Assoc Cancer Research* **37**:192.

30. Beham, A. W., and McDonnell, T. J. (1996) Bcl-2 confers resistance to androgen deprivation in prostate carcinoma cells. Proc Amer Assoc Cancer Research **37**:224.

31. Campos, L., Rouault, J.-P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J.-P., and Guyotat, D. (1993) High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* **81**:3091-3096.

32. Reber, U., Wullner, U., Trepel, M., Baumgart, J., Seyfried, J., Klockgether, T., Dichgans, J., and Weller, M. (1998) Potentiation of treosufan toxicity by the glutathione-depleting agent buthionine sulfoximine in human malignant glioma cells: the role of Blc-2. *Biochem Pharmacol* **55**:349-359.

33. Halliwell, B., and Gutteridge, J. M. C. (1985) Oxygen radicals and the nervous system. *TINS* **8**:22-26.

34. Maeda, H. (1994) The clinical effects of neocarzinostatin and its polymer conjugate, SMANCS. In: *Enediyne Antibiotics as Antitumor Agents* Marcel Decker, NY, NY, pp.363-381.

35. Chin, D.-H., Zeng, C.-H., Costello, C. E., and Goldberg, I. H. (1988) Sitesin the diyne-ene bicyclic core of neocarzinostatin chromophore responsible for hydrogen abstraction from DNA. *Biochemistry* **27**:8106-8114.

INDEX TERMS

apoptosis

Bcl-2

glutathione

neocarzinostatin

thiols